Discovery of salt-loving pleolipoviruses infecting archaea: vesicle-like virion is the key to success

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ACADEMIC DISSERTATION

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“Exciting discoveries await those who take the third way.”
Thorsten Allers and Moshe Mevarech, 2005

LIST OF ORIGINAL PUBLICATIONS

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ABBREVIATIONS – GENERAL

AM	ammonium molybdate
ATPase	adenosine-5'-triphosphatase
cryo-EM	cryo-electron microscopy
DNA	deoxyribonucleic acid
ds	double stranded
EM	electron microscopy
ESCRT	endosomal sorting complex required for transport
HGT	horizontal gene transfer
ICTV	International Committee on Taxonomy of Viruses
LUCA	last universal common ancestor
MCP	major capsid protein
nt	nucleotide
ORF	open reading frame
PEG	polyethylene glycol
PFU	plaque-forming unit
PG	phosphatidylglycerol
PGP-Me	phosphatidylglycerophosphate methyl ester
PGS	phosphatidylglycerosulfate
RNA	ribonucleic acid
rRNA	ribosomal RNA
S-DGD	sulfated diglycosylglycerol diether
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S-layer	surface layer
ss	single stranded
SSU	small subunit
TLC	thin-layer chromatography
TM	transmembrane
UA	uranyl acetate
VLP	virus-like particle
VP	virion protein

ABBREVIATIONS – VIRUSES AND VIRUS-LIKE PARTICLES

A3-VLP	*Methanococcus voltae* A3 virus-like particle
ABV	*Acidianus* bottle-shaped virus
ACV	*Aeropyrum* coil-shaped virus
AFV1	*Acidianus* filamentous virus 1
APOV1	*Aeropyrum pernix* ovoid virus 1
APOV2	*Aeropyrum pernix* ovoid virus 2
APBV1	*Aeropyrum pernix* bacilliform virus 1
APSV1	*Aeropyrum pernix* spindle-shaped virus 1
ASV1	*Acidianus* spindle-shaped virus 1
ATV	*Acidianus* two-tailed virus
HGPV-1	*Halogeometricum* pleomorphic virus 1
Hh-1	*Halobacterium halobium* (i.e. *salinarum*) virus 1
HHIV-2	*Haloarcula hispanica* icosahedral virus 2
HHPV-1	*Haloarcula hispanica* pleomorphic virus 1
His1	*Haloarcula hispanica* virus 1
His2	*Haloarcula hispanica* virus 2
HRPV-1	*Halorubrum* pleomorphic virus 1
HRPV-2	*Halorubrum* pleomorphic virus 2
HRPV-3	*Halorubrum* pleomorphic virus 3
HRPV-6	*Halorubrum* pleomorphic virus 6
HVTV-1	*Haloarcula vallismortis* tailed virus 1
Hs1	*Halobacterium salinarum* 1 virus
HSTV-2	*Haloarcula sinaiiensis* tailed virus 2
Mimivirus	Mimicking microbe virus
PAV1	*Pyrococcus abyssi* virus 1
PSV	*Pyrobaculum* spherical virus
SH1	Serpentine Lake *hispanica* virus 1
SIRV1	*Sulfolobus islandicus* rod-shaped virus 1
SIRV2	*Sulfolobus islandicus* rod-shaped virus 2
SNDV	*Sulfolobus neozealandicus* droplet-shaped virus
SSV1	*Sulfolobus* spindle-shaped virus 1
SSV6	*Sulfolobus* spindle-shaped virus 6
STIV	*Sulfolobus* turreted icosahedral virus
STIV2	*Sulfolobus* turreted icosahedral virus 2
STSV1	*Sulfolobus tengchongensis* spindle-shaped virus 1
TMV	Tobacco mosaic virus
TPV1	*Thermococcus prieurii* virus 1
TTSV1	*Thermoproteus tenax* spherical virus 1
TTV1	*Thermoproteus tenax* virus 1
VTA	*Methanococcus voltae* transfer agent

DEFINITIONS

The following definitions are based on Allers and Mevarech, 2005; Cann, 2005; Cavicchioli, 2011; DasSarma and DasSarma, 2012; and Rothschild and Mancinelli, 2001.

Extremophile is an organism that is dependent on extreme habitats like hypersaline or hyperthermic.

Acidophile is an organism that requires a low pH to grow, usually below 3.

Neutrophile is an organism that grows optimally at pH around 7.

Alkaliphile is an organism that requires a high pH to grow, usually above 9.

Mesophile is an organism that grows optimally at 15-60°C.

Thermophile is an organism that grows optimally above 60 and up to 80°C.

Hyperthermophile is an organism that grows optimally above 80°C.

Halophile is an organism that requires at least 0.17 M NaCl for optimal growth.

Methanogen is an anaerobic organism which produces methane by reduction of carbon dioxide, acetic acid or other, often simple, carbon compounds.

SUMMARY

Extremophiles are found in all three domains of cellular life but especially archaea are able to withstand harsh conditions. Halophilic archaea thrive in hypersaline environments like salt lakes and salterns which have been shown to contain high abundance of virus-like particles. So far, head-tailed viruses are the most common isolates infecting haloarchaea, which is in contrast to a variety of morphologies described for the viruses of hyperthermophilic archaea. Altogether, approximately 100 archaeal viruses have been isolated but only a fraction of them has been subjected to detailed structural analyses.

In this thesis, a novel haloarchaeal virus, *Halorubrum* pleomorphic virus 1 (HRPV-1), was isolated from a solar saltern. This virus was shown to have a flexible, pleomorphic vesicle-like virion devoid of a rigid protein capsid. The genome analyses revealed that HRPV-1 is the first archaeal virus to be isolated which does not have a double-stranded but a single-stranded DNA genome. A genomic region of HRPV-1 showed similarity to the genome of another haloarchaeal virus, *Haloarcula hispanica* virus 2 (His2), as well as to the genome of *Haloarcula marismortui* and *Natronomonas pharaonis* indicating that HRPV-1-like elements are widespread. Consistent with this, pleomorphic viruses resembling HRPV-1 and infecting haloarchaea of the genera *Haloarcula*, *Halorubrum* and *Halogeometricum* have subsequently been isolated from geographically distant locations, and this study was extended to altogether seven viruses. All these viruses were sensitive to lowered ionic strength confirming their halophilic nature. Based on the virion properties, these haloviruses were defined as pleolipoviruses.

Life-cycle studies showed that the pleolipoviruses are nonlytic and progeny virions are produced continuously resulting in host growth retardation. The most likely exit mechanism is budding which is consistent with the observation that the pleolipoviruses acquire their lipids unselectively from the host lipid pool. All pleolipoviruses have two major structural protein species, and biochemical dissociation studies showed that the larger-sized proteins form spike-like protrusions on the virion surface and the smaller-sized proteins are embedded in the inner surface of the membrane vesicle. The three-dimensional virion structure of HRPV-1 revealed that the spike structures are randomly distributed on the virion surface. The genome of the pleolipoviruses is enclosed in a lipid vesicle without associated nucleoproteins. Although the pleolipoviruses have different genome types, single- or double-stranded, circular or linear DNA, the membrane vesicle-based virion architecture is conserved.

This work introduced a novel group of pleomorphic viruses infecting extremely halophilic archaea and showed that vesicle-like virion architecture is common in hypersaline environments. Interestingly, the archaeal pleolipoviruses were observed to share several similarities with a bacterial mycoplasmavirus indicating that these viruses may form a viral lineage with an ancient origin.

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1. INTRODUCTION

Invisible to the naked eye, most abundant biological entities on Earth, complex molecular machines, poisons; all that is a virus. In 1885, Louis Pasteur used the Latin term for poison, *virus*, to describe an infective agent in his vaccination experiments. Today, viruses are defined as obligate intracellular parasites which multiply inside infected cells and produce viral particles (Cann, 2005). Viruses do not have ribosomes or energy metabolism, and these features distinguish them from cellular organisms (Cann, 2005). However, viruses are tightly connected to the cellular life as they infect all types of organisms, from multicellular plants and animals to unicellular bacteria and archaea.

1.1. Three domains of cellular life

1.1.1. Overview

Organisms have traditionally been divided into prokaryotes and eukaryotes (Murray, 1974). This represents, however, more a cellular-organization based division than a real phylogenetic classification. Thus, molecular features have become increasingly important and more relevant in classification compared to phenotypic criteria and present taxonomy relies primarily on small-subunit ribosomal ribonucleic acid (SSU rRNA) gene sequences, 16S and 18S rRNA (Pace, 2009; Woese et al., 1990).

In the late 1970s, 16S rRNA analysis of a group of methanogenic prokaryotes indicated that these organisms form a consistent phylogenetic class not related to bacteria (Fox et al., 1977). Consequently, it was proposed that methanogens represent a third line of life, *Archaeabacteria*, and this tripartite classification was to replace the traditional prokaryote-eukaryote dichotomy (Woese and Fox, 1977). Next, archaeabacteria were extended to include also some thermoacidophilic and extremely halophilic organisms (Woese et al., 1978). In the early 1990s, the sequence signatures of SSU rRNA were then used to establish the currently known three domains of cellular life, *Archaea*, *Bacteria* and *Eukarya* (Fig. 1) (Winker and Woese, 1991; Woese et al., 1990).

Informational proteins involved in deoxyribonucleic acid (DNA) replication, transcription and translation are less subject to horizontal gene transfer (HGT) than operational genes working in house-keeping and are thus considered to better reflect the evolutionary history of organisms (Jain et al., 1999; Rivera et al., 1998). Although archaea and bacteria resemble each other at a cellular level and share metabolic features (Krieg, 2001), the informational proteins of archaea are often more similar to those of eukaryotes than to their bacterial counterparts (Allers and Mevarech, 2005; Forterre et al., 2002). For example, X-ray crystallography revealed the highly similar structures of archaeal and eukaryotic RNA polymerases utilized in transcription (Hirata et al., 2008), and many archaea use eukaryotic-like histones in condensing DNA (Brochier-Armanet et al., 2011). This and phylogenetic comparisons based on rRNA sequences have implied that the closest relatives of archaea are eukaryotes (Forterre et al., 2002; Pace, 2009).

On one hand, archaeal genomes are mosaics of bacterial-like operational and eukaryotic-like informational genes, on the other a significant fraction of archaeal genes cannot be found in either bacteria

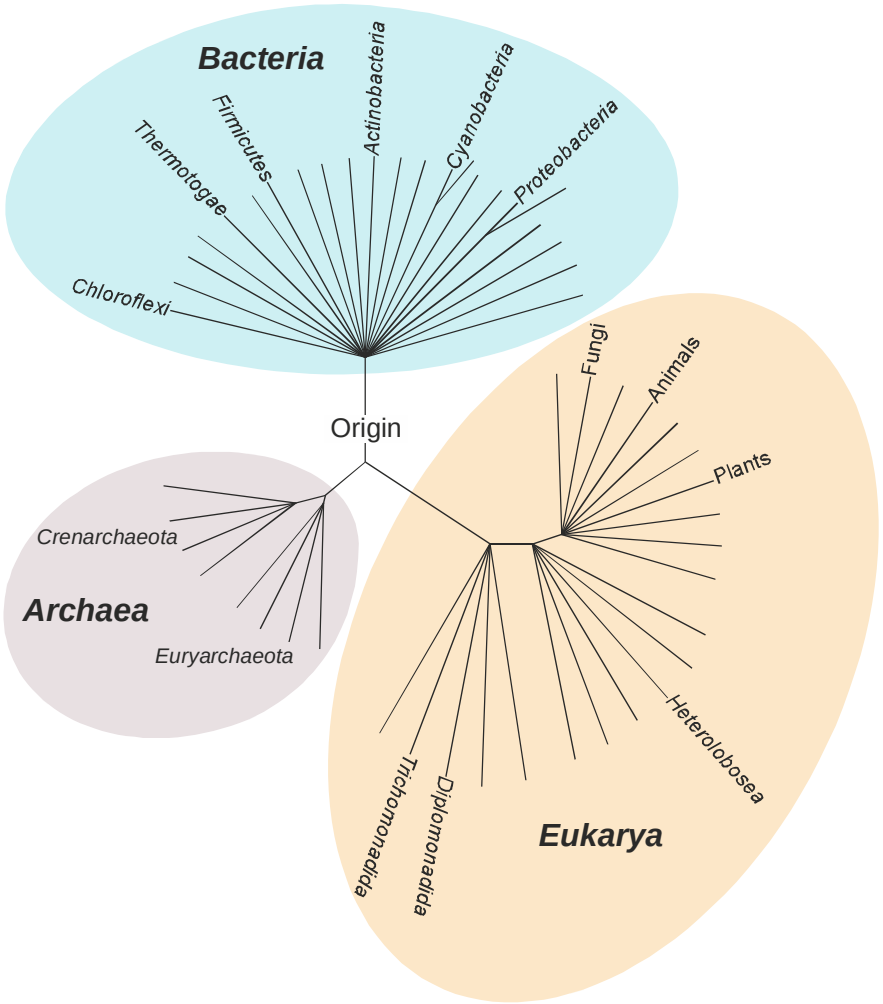

Figure 1. Three-domain tree of life based on rRNA sequences. The origin lies on the line leading to bacteria. Only a part of the major taxa is specified in each domain. Modified from Pace, 2009.

or eukaryotes emphasizing pathways and functions specific for archaea (Allers and Mevarech, 2005). Domain-specific features are summarized in Table 1. Ether-linked isoprenoid lipids are one of the archaeal signature traits (Boucher et al., 2004). Some archaeal cells display extraordinary shapes like the square cells of *Haloquadratum walsbyi* (Burns et al., 2007), and no bacterial peptidoglycan is found in archaeal cell walls (Albers and Meyer, 2011). Methanogenesis is restricted to the organisms of the domain *Archaea* (Krieg, 2001). Yet another feature separating archaea from bacteria and eukaryotes is pathogenicity. Although archaea inhabit human and other animals, no pathogenic isolates have been found (Gill and Brinkman, 2011).

Table 1. Signature features of organisms from different domains. Adapted from Cavicchioli, 2011.

Feature	*Archaea*	*Bacteria*	*Eukarya*
Side chains of lipids	Isoprenoid	Fatty acid	Fatty acid
Carbon linkage of lipids	Ether	Ester	Ester
Phosphate backbone of lipids	Glycerol-1-phosphate	Glycerol-3-phosphate	Glycerol-3-phosphate
Core transcription apparatus	Eukaryotic-like	Bacterial	Eukaryotic
Translation elongation factors	Eukaryotic-like	Bacterial	Eukaryotic
Nucleus and other organelles	No	No	Yes
Metabolism	Bacterial-like	Bacterial	Eukaryotic
Methanogenesis	Yes	No	No
Pathogenesis	No	Yes	Yes

1.1.2. Domain *Archaea*

There has been a growing interest towards the domain *Archaea* illustrated by an exponential increase in the number of sequenced archaeal genomes during the last few years. Over 100 archaeal genomes have now been sequenced (Brochier-Armanet et al., 2011). Notably, several important breakthroughs have been made within the domain *Archaea*. The first atomic resolution structure for a ribosome came from haloarchaea (Ban et al., 2000), and the 22nd amino acid, pyrrolysine, was found from methanogens (Hao et al., 2002). Furthermore, extremophilic archaea can be used to study how different cellular processes are adapted to harsh conditions, and they are also excellent model organisms for studying the origin of life. In addition, archaea and their cellular components are of interest in the development of biotechnological applications (Cavicchioli, 2011).

The domain *Archaea* has traditionally been divided into two major phyla, *Crenarchaeota* and *Euryarchaeota*. They include diverse thermophiles, hyperthermophiles, halophiles, and methanogens (Winker and Woese, 1991; Woese et al., 1990). In addition, several other phyla have been proposed. *Korarchaeota* was suggested to be an ancient archaeal lineage diverging before *Crenarchaeota* and *Euryarchaeota* (Barns et al., 1996) but korarchaea may also represent an early branch of *Crenarchaeota* (Cavicchioli, 2011; Pace, 2009). The isolation of a small-sized hyperthermophilic archaeon, *Nanoarchaeum equitans*, led to the proposal of the phylum *Nanoarchaeota* (Huber et al., 2002). However, this tiny archaeon more likely represents the phylum *Euryarchaeota* (Brochier-Armanet et al., 2011; Cavicchioli, 2011). The proposed phylum *Thaumarchaeota* contains mesophilic and thermophilic ammonia-oxidizing archaea which are widespread in nature (Brochier-Armanet et al., 2008).

Archaea are found in diverse extreme habitats which represent the environmental limits to life on Earth like hot springs or salt lakes. Although initially thought to be restricted to such environments, archaea have now been detected in a variety of nonextreme niches (Chaban et al., 2006). Cultivation-independent studies have shown the abundance of archaea but they have also been isolated and cultivated from moderate, both marine and soil, habitats (Chaban et al., 2006; Könneke et al., 2005; Tourna et al., 2011). Furthermore, it has been estimated that archaeal cells contribute to a significant fraction of Earth's biomass (DeLong and Pace, 2001).

1.2. Extremophilic archaea

Organisms living in extreme environments are called extremophiles, and they are found in all three domains of life (Cavicchioli, 2011; Rothschild and Mancinelli, 2001). However, especially archaea are known to thrive in various extreme conditions (Chaban et al., 2006). Temperature can range from less than 10 to more than 100°C in their habitats (Blöchl et al., 1997; Franzmann et al., 1988). Archaea are also found in hypersaline environments where NaCl concentration may reach saturation level, approximately 5.5-6.5 M (Bowers and Wiegel, 2011). In addition, archaea tolerate both acidic and highly alkaline conditions, elevated pressure, and anaerobic conditions (Marteinsson et al., 1999; Prokofeva et al., 2000; Xu et al., 2001). Extremophiles are not phylum specific since some are found in both *Euryarchaeaota* and *Crenarchaeaota*, and some organisms, like acidophilic hyperthermophiles, have several extremophilic features (Chaban et al., 2006; Rothschild and Mancinelli, 2001).

1.2.1. Hyperthermophiles

Hyperthermophiles, whose optimum growth temperature is above 80°C, belong to the domains *Archaea* and *Bacteria* (Rothschild and Mancinelli, 2001). Among archaea, hyperthermophiles are widely spread exemplified by the crenarchaeal orders *Desulfurococcales*, *Thermoproteales* and *Sulfolobales* and euryarhaeal *Archaeoglobales*, *Thermococcales* and *Methanopyrales*. Furthermore, all current crenarchaeal orders contain thermo- or hyperthermophiles (Chaban et al., 2006; Gribaldo and Brochier-Armanet, 2006).

Hyperthermophilic archaea live in diverse terrestrial and aquatic high-temperature habitats which are usually anaerobic or have very low oxygen concentrations. These can generally be found near volcanically active areas and include hot springs, marine hydrothermal vents, oil reservoirs, and acid mine drainages (Chaban et al., 2006). Hydrothermal vents provide a habitat supporting a great archaeal diversity and may even represent the origin of the last common archaeal ancestor (Auguet et al., 2010). It has been speculated that current archaea may descend from a hyperthermophilic ancestor as hyperthermophilic archaea are located at the base of the SSU rRNA phylogenetic trees (Forterre et al., 2002; Gribaldo and Brochier-Armanet, 2006).

Hyperthermophilic archaea hold the records for the highest growth temperature. A certain crenarchaeal strain has been observed to grow even at 121°C, the temperature used for autoclaving (Kashefi and Lovley, 2003). In addition, the crenarchaeon *Pyrolobus fumarii* is one of the most hyperthermophilic organisms known so far as it grows at 113°C and the lower limit for its growth is around 90°C (Blöchl et al., 1997). A thermostable DNA polymerase, Pfu, which is a commonly used enzyme in molecular biology, has been isolated from a hyperthermophilic crenarchaeon, *Pyrococcus furiosus*, emphasizing the biotechnological importance of archaea (Lundberg et al., 1991).

1.2.2. Methanogens

Methanogens are anaerobic organisms producing methane by the reduction of carbon dioxide, acetic acid, methanol, and other, often simple, carbon compounds (Cavicchioli, 2011). Besides being anaerobic, some methanogens are

halophilic or hyperthermophilic (Kurr et al., 1991; Lai and Gunsalus, 1992). All known methanogens are found in the domain *Archaea*, and they belong to the phylum *Euryarchaeota* (Krieg, 2001). Methanogenesis originated early in the domain *Archaea* but whether or not the last common archaeal ancestor was a methanogen is still unclear (Gribaldo and Brochier-Armanet, 2006).

Methanogens have been isolated from diverse environments like swamps, hot springs, freshwater and marine sediments, and they also inhabit animals (Chaban et al., 2006). *Methanobrevibacter smithii* is for example a common isolate in human intestinal flora (Eckburg et al., 2005). The first sequenced archaeal genome, and the fourth sequenced cellular genome, was that of *Methanocaldococcus jannaschii*, and this enabled for the first time the comparison of complete genomes from all three domains of life (Bult et al., 1996).

1.2.3. Halophiles

Salts are necessary for all organisms but halophiles require high salt concentrations for growth and thus thrive in saline environments (DasSarma and DasSarma, 2012). Depending on the salinity requirements, such organisms can be divided into different groups (Table 2). In addition to halophiles, there are halo-tolerant organisms which tolerate high salinity but do not require that for optimal growth (Bowers and Wiegel, 2011; DasSarma and DasSarma, 2012). Nonhalophilic organisms grow optimally at NaCl concentrations below 0.17 M (DasSarma and DasSarma, 2012).

In contrast to hyperthermophiles or methanogens, halophiles are found in all three domains of life. In addition to archaea and bacteria, a diversity of eukaryotes like green algae, diatoms, protozoa, yeasts and other fungi, brine flies and shrimps live at high salinity (Boetius and Joye, 2009; Rothschild and Mancinelli, 2001). In hypersaline environments salinity is higher than that of seawater, which is about 0.6 M of total dissolved salts. Despite the variety of halophiles, halophilic archaea usually dominate in the most saline environments (DasSarma and DasSarma, 2012; Oren, 2002). Halophilic archaea are classified into the phylum *Euryarchaeota*, and although some methanogens are halophilic, the majority belong to the order *Halobacteriales* of the class *Halobacteria* (Grant et al., 2001; Oren, 2012). From now on, halophilic archaea or haloarchaea refer to the archaea within *Halobacteriales*.

Table 2. Salt requirements of halophiles. Adapted from DasSarma and DasSarma, 2012.

Group	NaCl concentration for optimal growth	
	M (mol/l)	% (w/v)
Slight halophiles	0.17-0.85	1-5
Moderate halophiles	0.85-3.40	5-20
Extreme halophiles	3.40-5.10	20-30

1.2.3.1. Order *Halobacteriales*

The order *Halobacteriales* contains one family, *Halobacteriaceae*. This family was established in 1974 and at that time included only two genera, *Halobacterium* and *Halococcus*. By the end of 2011, altogether 36 genera and 129 species have been introduced illustrating the current diversity of the family (Oren, 2012). *Halobacterium* is the type genus but *Halorubrum* is the largest one in terms of the number of described species (Grant et al., 2001; Oren, 2012).

Most haloarchaea grow aerobically at neutral pH and are mesophilic, but slightly thermophilic as well slightly acidophilic and strongly alkaliphilic haloarchaea are also known (Bowers and Wiegel, 2011; DasSarma and DasSarma, 2012). Haloalkaliphiles require high pH and magnesium ions in addition to high NaCl concentration (Xu et al., 2001). Majority of haloarchaea are extreme halophiles (see Table 2) and grow optimally at 3.5-4.5 M NaCl (Grant et al., 2001). Besides, most of them are able to grow at concentrations higher than 5.0 M NaCl and some even in saturated salt conditions (Bowers and Wiegel, 2011). Haloarchaea growing at rather low salinities have been isolated from salt-marsh sediments, but also these required high NaCl concentrations for the optimal growth (Purdy et al., 2004).

Cells living in hypersaline environments must withstand a high osmotic pressure, and most bacterial and eukaryotic halophiles accumulate organic solutes like amino acids and their derivates to compensate this pressure (Roessler and Müller, 2001). In contrast, the majority of haloarchaea use high intracellular ion concentrations to balance the extracellular hypersalinity, and their cytoplasm typically contains high concentrations of potassium and chloride ions (Oren, 2006; Roessler and Müller, 2001). In addition, a strong envelope protects haloarchaeal cells. Most haloarchaea have a rigid surface layer (S-layer) which is composed of glycoproteins (Albers and Meyer, 2011; Oren, 2006). However, this proteinaceous S-layer is not found in coccoid-shaped haloarchaea, and for example *Halococcus* cells have a complex polysaccharide cell envelope. Unlike the glycoprotein S-layer, the cell walls of coccoid haloarchaea are stable in the absence of high salinity (Oren, 2006).

The cytoplasmic membrane of haloarchaea is a bilayer formed by diether lipids. Polar lipids are the major membrane lipids and include phospholipids, glycolipids and sulfolipids. The most abundant polar lipids are phosphatidylglycerol (PG) and phosphatidylglycerophosphate methyl ester (PGP-Me) as well as phosphatidylglycerosulfate (PGS) in certain neutrophilic species (Oren, 2006). Membranes of haloarchaea are stable at high salinities, and it has been shown that PGP-Me accounts for 50-80% of the polar lipids and helps to stabilize the membranes in hypersaline environments (Tenchov et al., 2006). Cultures of haloarchaea are typically orange-red due to carotenoid pigments, which are neutral lipids in their cell membranes. All neutral or nonpolar lipids account for only 10% of the total membrane lipids (Oren, 2006).

1.2.3.2. Genera *Haloarcula*, *Halorubrum* and *Halogeometricum*

Haloarcula, *Halorubrum* and *Halogeometricum* cells are characteristically pleomorphic, although rod-shaped cells are common and even square- and triangle-shaped representatives are known (Grant et al., 2001; Oren, 2006). The genera *Haloarcula* and *Halogeometricum* were both established as a result of the isolation and characterization of new haloarchaeal strains from solar salterns (Montalvo-Rodríguez et al., 1998; Torreblanca et al., 1986). In contrast, the genus *Halorubrum* was established when four *Halobacterium* species were observed to form a distinct phylogenetic group based on the 16S rRNA gene sequences and reassigned to a new genus (McGenity and Grant, 1995). Unlike *Haloarcula* and *Halorubrum*, the genus *Halogeometricum* contains only a few described species (Cui et al., 2010; Montalvo-Rodríguez et al., 1998).

Most of the species in the genera *Haloarcula*, *Halorubrum* and *Halogeometricum* have their optimum NaCl concentration above 3 M (Bowers and Wiegel, 2011; Grant et al., 2001; Oren, 2006). In addition, some *Halorubrum* and *Halogeometricum* species require high

magnesium concentration for the optimal growth, some up to 1.2 M Mg^{2+} (Grant et al., 2001; Oren, 2006). The genus *Halorubrum* contains also many alkaliphiles (Bowers and Wiegel, 2011). Temperature tolerance of these haloarchaea is wide, from 4 to 56°C, although the optimal growth typically occurs between 30 and 50°C (Bowers and Wiegel, 2011; Grant et al., 2001; Oren, 2006). *Halorubrum lacusprofundi* was isolated from a hypersaline Antarctic lake and is thus able to grow at low temperature (Franzmann et al., 1988).

Representatives of *Haloarcula*, *Halorubrum* and *Halogeometricum* are also characterized at the genomic level as at least one complete genome sequence from each group is available in public databases. For example, *Haloarcula hispanica* contains two chromosomes, one main chromosome and one minichromosome, and a megaplasmid (Liu et al., 2011).

1.2.3.3. Hypersaline environments and haloarchaea

Hypersaline environments are found all over the planet, and although salinity is a significant factor limiting life, these habitats support growth of dense microbial populations (Oren, 2002). The main habitat types of haloarchaea include salt lakes like the Dead Sea in Israel, the Great Salt Lake in Utah and Antarctic hypersaline lakes. Haloarchaea are also found in soda lakes, such as Lake Magadi in Kenya, where pH is usually above 10 due to sodium carbonate. Artificial solar salterns are as well inhabited by haloarchaea. Besides aquatic environments, haloarchaea have been isolated from saline soil, salted food products and ancient saline deposits (Chaban et al., 2006; DasSarma and DasSarma, 2012). Although all these habitats are characterized by high NaCl concentration, other ions can also be abundant. For example in the Dead Sea magnesium level exceeds that of sodium (Chaban et al., 2006; Oren, 2002).

Solar salterns are multipond systems where seawater is evaporated in a series of connected shallow ponds. The pond, where NaCl precipitates, is called the crystallizer (Benlloch et al., 2001; Ochsenreiter et al., 2002). Halophilic bacteria are abundant and may account for up to one-fourth of prokaryotes in the crystallizer ponds (Antón et al., 2000). However, haloarchaea dominate in these ponds, and cultivation-independent studies, both 16S rRNA gene sequencing and fluorescence *in situ* hybridization, have shown that different haloarchaeal species dominate in different crystallizers. For example, *Halorubrum*, *Halobacterium* and *Haloquadratum* cells can each be a dominant population in different ponds (Antón et al., 1999; Benlloch et al., 2001; Bidle et al., 2005; Pašić et al., 2007). Thus, although the crystallizer ponds share the high salinity, local environmental conditions have a key role in shaping their haloarchaeal communities.

A number of haloarchaeal species has been isolated from the crystallizer ponds, but representatives from the genera *Halorubrum*, *Halobacterium*, *Haloferax*, and *Haloarcula* are most commonly isolated (Benlloch et al., 2001; Bidle et al., 2005; Pašić et al., 2007). In a global study of archaeal 16S rRNA gene sequences, it was observed that hypersaline habitats still contain a large fraction of uncultivated species (Auguet et al., 2010). The cultivability of haloarchaea varies from one habitat to another. In some hypersaline environments all main haloarchaeal groups are cultivable and in others cultivation and molecular methods give different species ranges (Burns et al., 2004; Ochsenreiter et al., 2002), and it seems that the cultivability depends on the haloarchaeal composition of a particular habitat.

1.3. Virus world – virosphere

Viruses can be found, at least almost, everywhere, and the viral proportion of the biosphere is called virosphere (Comeau et al., 2008; Suttle, 2007). It has been estimated that Earth's oceans contain about 10^{30} virus particles (Suttle, 2007). As the viral abundance and diversity in soil are comparable to and sometimes exceed those of aquatic environments, the total number of viruses in the biosphere is even higher (Comeau et al., 2008; Srinivasiah et al., 2008). Altogether this means that viruses outnumber cellular organisms at least 10- or 15-fold (Bamford, 2003; Suttle, 2007).

Viruses are the most abundant nucleic-acid-containing entities on Earth but they represent only a minor fraction of Earth's biomass (Suttle, 2007). This is due to the small size of viral particles. One of the largest viruses known to date, the Mimivirus (for mimicking microbe), has a fiber-covered capsid of 750 nm in diameter (Klose et al., 2010). In comparison, some of the smallest viruses have diameters of about 20 nm (Ritchie et al., 1989).

Due to the abundance and diversity of viruses, practically every organism is susceptible for a viral infection and thus viruses play major roles in regulating cellular life. Furthermore, viruses are causative agents of several, both mild and severe, infectious diseases (Jones, 2009; Yang et al., 2008). Currently, most of the emerging and re-emerging infectious diseases are of viral origin because viruses are highly variable (Yang et al., 2008). Viruses are also one of the leading causes of microbe mortality in the oceans. They are approximated to kill 20% or more of the microbial biomass every day and therefore affect both bio- and geochemical cycles. Viruses might also play a significant role in climate change by affecting cellular life in the oceans, and vice versa, climate change may affect viruses through their host populations (Danovaro et al., 2011; Suttle, 2007).

Viruses are entirely dependent on their host cells since they lack ribosomes and energy metabolism and thus can replicate only inside the cells (Cann, 2005). The key factor differentiating viruses from other self-replicating genetic elements like plasmids is their ability to build an infective virus particle encapsulating the genetic material (Krupovič and Bamford, 2010). Thus, it has been proposed that viruses should be defined as capsid-encoding organisms while cells are defined as ribosome-encoding organisms (Raoult and Forterre, 2008).

1.3.1. Virion morphologies

Particles, which resemble viruses based on electron microscopy (EM) observations, are often assigned as virus-like particles (VLPs) (Børsheim et al., 1990; Suttle, 2007). The word virion, however, refers to a mature, infectious virus particle. The function of a virion is to protect the viral genome and to deliver it from one host cell to another. All virions are composed of two main structural components, nucleic acids and proteins. In addition, lipids are an important structural part of certain virion types (Cann, 2005). Based on the virion architectures, virions can be roughly divided into four morphotype categories: 1) icosahedrally symmetric, 2) helically symmetric, 3) combination of icosahedral and helical symmetries, and 4) partially symmetric and asymmetric (Fig. 2).

Icosahedrally-symmetric virions, which are composed of 20 triangular facets forming an icosahedron, are encountered among viruses infecting archaea, bacteria and eukaryotes (King et al., 2012). In the simplest icosahedral virion, three copies of a capsid protein form the triangular facet and the total number of capsomers is 60,

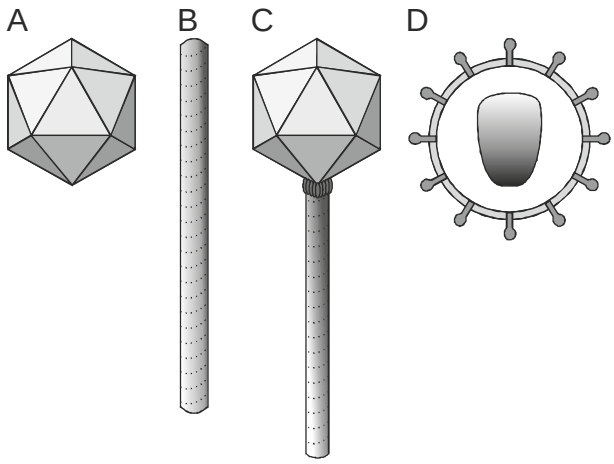

Figure 2. Schematic presentation of virion morphotypes. (A) Icosahedral symmetry. (B) Helical symmetry. (C) Combination of icosahedral and helical symmetries. (D) Enveloped asymmetric virion. The virions are not in scale. Based on King et al., 2012.

like in bacterial virus PhiX174 (McKenna et al., 1992). Helical symmetry is also common for viruses infecting hosts from all three domains of life, and helical morphotypes vary from rod-shaped to filamentous (King et al., 2012). A good example of helically-symmetric rod-shaped viruses is tobacco mosaic virus (TMV) which has played an important role already in the early phases of virology (Cann, 2005; Klug, 1999). A helical virion is formed when multiple copies of one capsid protein surround the viral genome, and the virion length depends on the genome size (King et al., 2012; Klug, 1999).

A head-tailed virion is formed when icosahedral and helical capsids are put together. Viruses with icosahedrally-symmetric heads and helical tails are known to infect only archaeal and bacterial cells, and in fact most of the described prokaryotic viruses (96%) belong to this group (Ackermann and Prangishvili, 2012; King et al., 2012). Instead of isometric capsids, some head-tailed viruses have elongated, prolate heads (Ackermann and Prangishvili, 2012).

Other known virion morphotypes range from pleomorphic to highly complex structures (King et al., 2012). Such viruses infect organisms from all domains of cellular life but especially archaeal and eukaryotic viruses have several unusual morphotypes (King et al., 2012; Pina et al., 2011).

Lipids found in the virions are host-derived, either from the cytoplasmic or cell organelle membranes (Cann, 2005). In enveloped virions (Fig. 2D), a lipid vesicle encloses a protein capsid or a nucleoprotein complex (Huiskonen and Butcher, 2007; Rossman and Lamb, 2011). A lipid vesicle can also be found inside a protein capsid (Huiskonen and Butcher, 2007). In addition to these lipid-containing viruses, there are even more complex membranous viruses such as vaccinia virus with two membrane layers (Grünewald and Cyrklaff, 2006).

1.3.2. Classification of viruses

Viral abundance and diversity are overwhelming. Consequently, a number of ways to classify viruses has been introduced. Lwoff classification from 1962 was developed using the physical properties of the virion (Lwoff and Tournier, 1966). This, already hierarchical, system included four main criteria. First, viruses were

divided into two groups based on their genetic material: DNA or RNA viruses. Next criterion was the symmetry of the capsid. The third division was made based on whether the nucleocapsid was enveloped or naked. At the time, a protein shell was also considered to be an envelope. Finally, viruses were classified into families using the diameter of a virion or the number of capsomers.

Baltimore classification is based on the nature of the nucleic acids in the virion as well as how viruses synthesize messenger RNA using their genetic material and how they replicate (Baltimore, 1971). In addition to two different nucleic-acid types, the viral genomes can be either circular or linear, single or double stranded (ss or ds), or as one molecule or segmented (Cann, 2005). According to the Baltimore system, viruses can be classified into seven classes of DNA, RNA and reverse-transcribing viruses. dsDNA and ssDNA viruses belong to classes I and II, respectively, and dsRNA viruses form class III. Classes IV and V contain positive-sense and negative-sense ssRNA viruses, respectively. Reverse transcribing positive-sense ssRNA viruses and dsDNA viruses form classes VI and VII, respectively (Baltimore, 1971; Summers and Mason, 1982).

International Committee on Taxonomy of Viruses (ICTV) is perhaps the most dominant classification authority at the moment. The first report was published in 1971 and the most recent, ninth report, in 2012 (King et al., 2012). Viruses are classified according to a hierarchical system into orders, families, genera, and species. The genome type and thus the Baltimore classification have an important role in the ICTV classification. Other main criteria are host organisms and virion morphotypes. Roughly, viruses are divided into archaeal, bacterial and eukaryotic viruses representing different morphotypes with DNA or RNA genomes. During 1995-2012, approximately 40 new viral families have been introduced and the number of orders has increased from one to six (Fig. 3A). However, the current viral orders contain only about one-fourth of all described viral families (Fig. 3B).

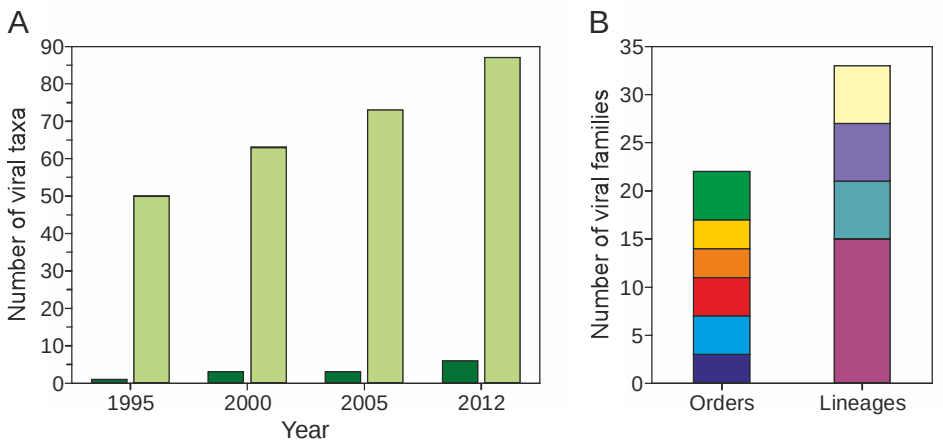

Figure 3. Virus classification. (A) Increasing number of viral orders (dark green) and families (light green) according to ICTV. (B) Viral families assigned to six orders and four lineages (indicated in different colours) according to Abrescia et al., 2012 and King et al., 2012.

1.3.3. Virus evolution and viral lineages

Genome sequence comparison is an important tool when studying relationships between viruses. However, viruses are old, perhaps older than the last universal common ancestor (LUCA) of cellular life, and furthermore viruses and their genome sequences evolve rapidly (Bamford, 2003; Bamford et al., 2002; Duffy et al., 2008; Krupovič and Bamford, 2010). Due to this, it is not possible to detect long-range evolution of viruses relying only on sequences. Thus, in order to detect evolutionary relationships across the whole virosphere and between viruses, which show no sequence similarity, other approaches are needed.

The importance of virion architecture and structure for viral classification was already appreciated by the Lwoff-classification system (Lwoff and Tournier, 1966). More recently, structural studies of icosahedrally-symmetric viruses have shown that viruses infecting hosts from different domains of cellular life can be classified into lineages which most likely have ancestors predating the LUCA. Remarkably, within a lineage, all viruses have the same major capsid protein (MCP) fold although they may not have any recognizable sequence similarity. In addition, viruses within the same lineage share many other features of virion architecture and assembly (Abrescia et al., 2012; Abrescia et al., 2010; Bamford, 2003; Bamford et al., 2002; Benson et al., 2004). This structure-based lineage classification represents a higher-level system compared to the ICTV and its virion-centered view reaches beyond sequence comparisons thus revealing deeper evolutionary relationships between viruses (Krupovič and Bamford, 2010).

The viral “self” structures and functions determine the lineage, and they are vertically inherited within the lineage (Bamford, 2003; Bamford et al., 2002; Krupovič and Bamford, 2007). The “self” elements include proteins which are essential for the virion architecture and assembly like an MCP and a genome-packaging adenosine-5'-triphosphatase (ATPase). In contrast, the viral “nonself” proteins, like those involved in replication and host-cell interaction, enable viruses to survive in changing environments and host systems. While such genes are likely to be exchanged by HGT, genes responsible for virion architecture and assembly are conserved (Abrescia et al., 2012; Abrescia et al., 2010; Bamford, 2003; Krupovič and Bamford, 2007; Saren et al., 2005).

So far, four different lineages have been established (Fig. 3B): picorna-, PRD1-adenovirus-, bluetongue virus-, and HK97-like lineages. All established lineages contain only viruses with icosahedrally-symmetric capsids, although some of these have a lipid envelope surrounding the capsid or a helical tail attached to the capsid. It has been proposed that these lineages will include the majority of all icosahedral viruses (Abrescia et al., 2012; Abrescia et al., 2010). Over one-third of the viral families have been assigned to the current lineages which is more than into the orders (Fig. 3B). Only nine families are found in both viral lineages and orders. Six families from the orders *Caudovirales* and *Herpesvirales* form the HK97-like lineage, and the picorna-like lineage contains three families from the orders *Tymovirales* and *Picornavirales* (Abrescia et al., 2012).

The PRD1-adenovirus- and HK97-like lineages are the only ones containing viruses infecting hosts from all three domains of life (Abrescia et al., 2010). Many striking similarities were initially detected between bacterial virus PRD1 and human adenovirus, and when it was found out that the MCPs of these viruses have the same fold, it was proposed that PRD1 and adenovirus are related (Benson et al., 1999). Soon after, the lineage was extended so that it included viruses infecting gram-negative and gram-positive bacteria, vertebrates and invertebrates, algae, and archaea (Bamford

et al., 2002; Benson et al., 2004).

The characteristic fold of the HK97-like lineage was first recognized in coliphage HK97 and has since been described for a number of bacterial head-tailed viruses (Abrescia et al., 2010; Wikoff et al., 2000). When the HK97-like fold was found in the icosahedral capsid of herpesviruses, the lineage was extended to eukaryotic viruses (Baker et al., 2005). For archaeal head-tailed viruses, the HK97-fold has been predicted to exist by structural modeling but no MCP structure has yet been solved (Krupovič et al., 2010).

1.4. Archaeal viruses

The first archaeal virus to be isolated was described almost 40 years ago, before the establishment of the domain *Archaea* (Torsvik and Dundas, 1974; Woese et al., 1990). However, wider attention towards archaeal viruses has developed quite recently (Pina et al., 2011; Prangishvili et al., 2006a; Prangishvili et al., 2006b). So far, approximately 100 viruses infecting archaea have been described in contrast to over 6000 characterized bacterial viruses (Ackermann and Prangishvili, 2012; Atanasova et al., 2012; Pina et al., 2011).

Despite the small number of isolates, one of the most interesting features of archaeal viruses is the diversity of virion morphotypes (Fig. 4). In addition, some of the morphotypes, including spindle-, bottle- and droplet-shaped virions, are not found among bacterial or eukaryotic viruses (Pina et al., 2011). Another peculiar thing is the genome type which is currently limited to DNA. The majority of the studied archaeal viruses have a dsDNA genome (Pina et al., 2011), and only one hyperthermophilic virus and a few halophilic viruses with ssDNA genomes have been reported (Mochizuki et al., 2012; Senčilo et al., 2012). However, viral RNA genomes have recently been detected in metagenomic analyses of archaea-dominated hot springs indicating that RNA viruses may also infect members of the domain *Archaea* (Bolduc et al., 2012).

The host range of the studied archaeal viruses is limited to extremophiles as all isolates infect either hyperthermophilic crenarchaea or hyperthermophilic, halophilic or methanogenic euryarchaea (Atanasova et al., 2012; Gorlas et al., 2012; Pina et al., 2011). However, it has been acknowledged that archaea are also abundant in moderate environments, and a putative provirus was recently detected in the genome of an ammonia-oxidizing archaeon belonging to the proposed phylum *Thaumarchaeota* (Krupovič et al., 2011). Nevertheless, viruses infecting nonextremophilic archaea still wait to be isolated.

1.4.1. Crenarchaeal and euryarchaeal viruses

Viral isolates infecting euryarchaea are more numerous but virion morphotypes are more diverse among crenarchaeal isolates (Fig. 4) (Atanasova et al., 2012; Pina et al., 2011). Accordingly, crenarchaeal viruses are currently classified into almost ten viral families including one recently proposed family, "*Spiraviridae*" (Ackermann and Prangishvili, 2012; Mochizuki et al., 2012; Mochizuki et al., 2010; Pina et al., 2011). In contrast, only six different morphotypes, including three types of head-tailed virions, have been described for euryarchaeal viruses (Fig. 4) (Atanasova et al., 2012; Pina et al., 2011).

Only two morphotypes are common for both crenarchaeal and euryarchaeal viruses, spindle shaped and tailless icosahedral (Fig. 4). Another major difference between these virus groups is the absence of head-tailed viruses infecting

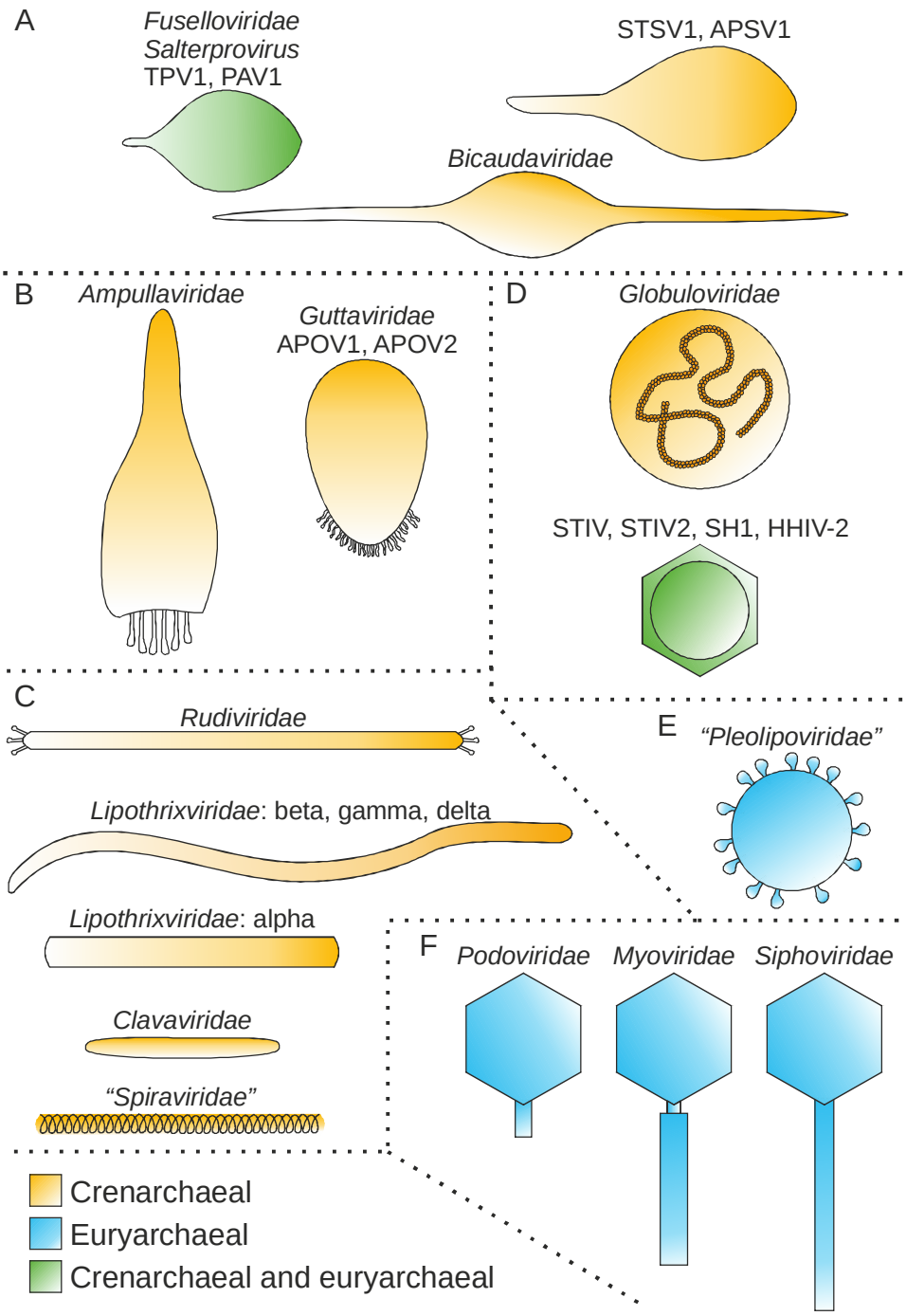

Figure 4. Virion morphotypes of creanarchaeal and euryarchaeal viruses. Individual viruses (see names in Abbreviations – viruses and virus-like particles) are indicated if they are not assigned to any viral family or genus. The virions are not drawn to scale. (A) Spindle-shaped virions. (B) Bottle- and droplet-shaped virions. (C) Linear virions. (D) Spherical virions. (E) Pleomorphic virions (see section 4.4.1.). (F) Head-tailed virions. Modified from Ackermann and Prangishvili, 2012; Mochizuki et al., 2012; and Pina et al., 2011.

crenarchaea while most of the euryarchaeal virus isolates are such (Atanasova et al., 2012; Pina et al., 2011). However, head-tailed VLPs have been observed in hyperthermic environments (Krupovič et al., 2010; Rachel et al., 2002) indicating that such viruses are not excluded from the environments where crenarchaea often dominate (Chaban et al., 2006).

In addition to the provirus from a thaumarchaeon (Krupovič et al., 2011), a variety of proviruses have been detected in both euryarchaeal and crenarchaeal genomes (Held and Whitaker, 2009; Krupovič and Bamford, 2008; Krupovič et al., 2010; Mochizuki et al., 2011). For example, proviruses related to head-tailed, tailless icosahedral, and spindle-shaped viruses have been found in the genomes of hyperthermophilic, halophilic and methanogenic archaea (Held and Whitaker, 2009; Krupovič and Bamford, 2008; Krupovič et al., 2010).

1.4.2. Viruses infecting hyperthermophiles

Viruses infecting hyperthermophilic archaea form a major archaeal virus group in addition to haloarchaeal viruses. Most of the described hosts belong to the phylum *Crenarchaeota*, typically to the genera *Sulfolobus* or *Acidianus*, and only a few viruses are known to infect hyperthermophilic euryarchaea (Gorlas et al., 2012; Pina et al., 2011). Persistent infections resulting in continuous virus production and host cell growth retardation are common among viruses infecting hyperthermophiles, and only a few lytic viruses have been reported. Virion morphotypes found among these viruses are spindle, bottle and droplet shaped as well as spherical and linear, and many of these viruses contain lipids (Pina et al., 2011). Spindle- and droplet-shaped viruses have circular and bottle-shaped viruses linear dsDNA genomes. Linear and spherical viruses have either circular or linear dsDNA genomes depending on a virus, except for a newly described linear virus with a circular ssDNA genome (Mochizuki et al., 2012; Pina et al., 2011; Prangishvili et al., 2006b).

1.4.2.1. Spindle-shaped viruses

Spindle-shaped viruses have so far been isolated only in the domain *Archaea*. These particles are commonly found in hyperthermic environments, and spindle-shaped viruses are known to infect both hyperthermophilic crenarchaea and euryarchaea (Gorlas et al., 2012; Pina et al., 2011; Rachel et al., 2002). Spindle-shaped virions are wider in the middle and taper towards the ends, and three virion types are recognized based on the tail structure (Fig. 4A). Particles can have one very short tail like *Sulfolobus* spindle-shaped virus 1 (SSV1), one long tail like *Sulfolobus tengchongensis* spindle-shaped virus 1 (STSV1) or two long tails like *Acidianus* two-tailed virus (ATV) (Martin et al., 1984; Prangishvili et al., 2006c; Xiang et al., 2005).

Crenarchaeal spindle-shaped viruses, which have one short tail with tail fibers, are classified into the family *Fuselloviridae*. At least ten fuselloviruses have been isolated, and they all infect hyperthermophilic crenarchaea of the genera *Sulfolobus* or *Acidianus* (King et al., 2012; Pina et al., 2011). The first isolate, and the type species of the family, is SSV1 (King et al., 2012; Martin et al., 1984). Although spindle shaped, this virion type is flexible. Some of the SSV1 virions are elongated, and there is also some size variation among the particles (Martin et al., 1984). In addition, *Sulfolobus* spindle-shaped virus 6 (SSV6) and *Acidianus* spindle-shaped virus 1 (ASV1) have rather pleomorphic virions (Redder et al., 2009).

The virion of SSV1 contains one major structural protein, VP1 (VP for virion protein), and two minor ones, VP2 and VP3. VP1 and VP3, which are highly homologous, are coat proteins while VP2 is most likely a DNA-binding protein (Reiter et al., 1987). In addition, two other minor viral proteins, C792 and D244, have been identified to be associated with the SSV1 particle (Menon et al., 2008). Circular dsDNA genomes of fuselloviruses share gene synteny and significant nucleotide sequence similarity. VP1- and VP3-encoding genes are found in all fuselloviruses, but VP2-encoding gene is found only in SSV1, SSV6 and ASV1. C792 homologues are present in all fusello-viruses, and D244 homologues are found in all but three fuselloviruses (Redder et al., 2009).

Only one spindle-shaped virus, *Thermococcus prieurii* virus 1 (TPV1), and one VLP, *Pyrococcus abyssi* virus 1 (PAV1), have been isolated from euryarchaea. TPV1 and PAV1 resemble morphologically fuselloviruses (Geslin et al., 2003; Gorlas et al., 2012). In addition, TPV1 and PAV1 share the genome type and size of fuselloviruses (Geslin et al., 2007; Gorlas et al., 2012; Redder et al., 2009). Like SSV1, PAV1 has only one major structural protein (Geslin et al., 2007). Due to many similarities, TPV1 and PAV1 could be classified into the family *Fuselloviridae*. However, they show no significant sequence similarity to fuselloviruses (Geslin et al., 2007; Gorlas et al., 2012). Furthermore, the sequence similarity between TPV1 and PAV1 is limited to two predicted gene products proposed to function in adsorption (Geslin et al., 2007; Gorlas et al., 2012).

Two-tailed virus ATV, which infects a hyperthermophilic crenarchaeon, is classified into the family *Bicaudaviridae* (King et al., 2012; Prangishvili et al., 2006c). Remarkably, ATV virions develop the long tails outside the host cells (Häring et al., 2005b; Prangishvili et al., 2006c). Crenarchaeal viruses STSV1 and *Aeropyrum pernix* spindle-shaped virus 1 (APSV1) have one long tail of variable length (Mochizuki et al., 2011; Xiang et al., 2005). However, two-tailed forms of STSV1 and APSV1 have also been observed (Mochizuki et al., 2011; Xiang et al., 2005), and thus it has been proposed that they could be classified into the family *Bicaudaviridae* (Pina et al., 2011). Interestingly, one of the major structural proteins of ATV shows significant similarity to the single major structural protein of STSV1 (Prangishvili et al., 2006c; Xiang et al., 2005). However, STSV1 has been reported to contain lipids in contrast to ATV where no lipids have been detected (Prangishvili et al., 2006c; Xiang et al., 2005).

1.4.2.2. Bottle- and droplet-shaped viruses

One of the unique, and most peculiar, shapes among archaeal viruses is that of the bottle shape (Fig. 4B). Crenarchaeal *Acidianus* bottle-shaped virus (ABV) is classified into the family *Ampullaviridae*, currently containing only this virus (Häring et al., 2005a; King et al., 2012). The broader end of the bottle-shaped ABV virion is covered by short filaments. An outer layer surrounds a cone-shaped structure most likely formed by a nucleoprotein filament, and it is proposed that a separate structural unit forms the narrow end, the tip of the bottle. At least six major protein constituents form this complex virion (Häring et al., 2005a).

In addition to bottle-shaped virions, droplet-shaped particles are unique for archaeal viruses (Fig. 4B), and they are found to infect crenarchaeal hosts only (Arnold et al., 2000; Mochizuki et al., 2011). Currently, *Sulfolobus neozealandicus* droplet-shaped virus (SNDV) is the only member in the family *Guttaviridae* but two other members, *Aeropyrum pernix* ovoid viruses 1 and 2 (APOV1 and APOV2), have been proposed (King et al., 2012; Mochizuki

et al., 2011). Ovoid-shaped particles of APOV1 and APOV2 resemble SNDV virions, except for that one pointed end of SNDV virions is densely covered by fibers (Arnold et al., 2000; Mochizuki et al., 2011). Only one major protein component forms the droplet-shaped virions of SNDV (Arnold et al., 2000).

1.4.2.3. Linear viruses

Linear viruses (Fig. 4C) are abundant in hyperthermic environments (Häring et al., 2005a; Rachel et al., 2002), and most of such archaeal viruses are classified into two families, *Lipothrixviridae* and *Rudiviridae* (King et al., 2012; Pina et al., 2011). These viruses have dsDNA genomes in contrast to the ssDNA and ssRNA genomes of linear bacterial and eukaryotic viruses, respectively (Pina et al., 2011). Both families contain several viral species, and based on the terminal structures of the virions and genome sequence similarities, lipothrixviruses are divided into four genera: *Alpha*-, *Beta*-, *Gamma*-, and *Deltalipothrixvirus* (King et al., 2012; Pina et al., 2011). The host range of rudi- and lipothrixviruses include only crenarchaea, from the genera *Acidianus*, *Stygiolobus*, *Sulfolobus*, and *Thermoproteus* (Pina et al., 2011).

The virions of lipothrixviruses are flexible lipid-containing filaments, except for the alphalipothrixvirus *Thermoproteus tenax* virus 1 (TTV1), which has enveloped nonflexible virions (King et al., 2012; Pina et al., 2011). The gammalipothrixvirus *Acidianus* filamentous virus 1 (AFV1) contains two major coat proteins. Both proteins have been shown to bind DNA, and the virion model of AFV1 suggests that the viral DNA wraps around one coat protein and the other protein, while interacting with the lipid envelope, binds to the DNA (Goulet et al., 2009).

The virions of rudiviruses are unenveloped rigid rods (Pina et al., 2011). *Sulfolobus islandicus* rod-shaped viruses 1 and 2 (SIRV1 and SIRV2) contain only one major coat protein, and this basic protein most likely binds to DNA and forms a helical structure (Prangishvili et al., 1999). The major coat proteins of rudiviruses and lipothrixviruses are structurally highly similar (Goulet et al., 2009; Prangishvili and Krupovič, 2012). Furthermore, these viruses share several homologous genes (Prangishvili and Krupovič, 2012). Thus, it has been proposed that the families of these linear archaeal viruses form the order *Ligamenvirales* (Prangishvili and Krupovič, 2012).

In addition to rudi- and lipothrixviruses, two new linear morphotypes have recently been described for the viruses of hyperthermophilic crenarchaea (Fig. 4C) (Mochizuki et al., 2012; Mochizuki et al., 2010). *Aeropyrum pernix* bacilliform virus 1 (APBV1) has stiff bacillus-like virions, and thus the virus represents a novel viral family, *Clavaviridae* (Ackermann and Prangishvili, 2012; Mochizuki et al., 2010; Pina et al., 2011). The APBV1 virions are composed of one major and three minor structural proteins (Mochizuki et al., 2010). *Aeropyrum* coil-shaped virus (ACV) has hollow cylindrical virions formed by a coiling nucleoprotein fiber, and due its unique properties it has been proposed to represent a new viral family, "*Spiraviridae*" (Mochizuki et al., 2012). Unlike rudi- and lipothrixviruses, which have linear genomes, APBV1 and ACV have circular genomes (Mochizuki et al., 2012; Mochizuki et al., 2010; Pina et al., 2011). Furthermore, ACV is so far the only ssDNA virus described for hyperthermophiles (Mochizuki et al., 2012).

1.4.2.4. Spherical viruses

There are two types of spherical viruses infecting hyperthermophilic crenarchaea, those classified into the viral family *Globuloviridae* and unclassified viruses with icosahedral symmetry (Fig. 4D). *Globuloviridae* contains two members with linear dsDNA genomes, *Pyrobaculum* spherical virus (PSV) and *Thermoproteus tenax* spherical virus 1 (TTSV1) (Ahn et al., 2006; King et al., 2012; Pina et al., 2011). The lipid envelope of globuloviruses encloses the genome which is in a helical nucleoprotein complex, and thus they resemble morphologically eukaryotic ssRNA paramyxoviruses (Häring et al., 2004). Three structural proteins of PSV and one structural protein of TTSV1 have been identified (Ahn et al., 2006; Häring et al., 2004).

Two tailless icosahedral viruses, *Sulfolobus* turreted icosahedral virus (STIV) and STIV2, have been described to infect the crenarchaea of the genus *Sulfolobus*. STIV and STIV2 are closely related. Their virions contain at least nine structural proteins and an inner lipid membrane surrounding a circular dsDNA genome (Happonen et al., 2010; Maaty et al., 2006; Rice et al., 2004). STIV, which belongs to the PRD1-adenovirus-like lineage, has been analysed from the transcriptomics to the structural level and thus is one of the most extensively studied archaeal viruses and a model virus for hyperthermophilic crenarchaea (Benson et al., 2004; Fu and Johnson, 2012). STIV and rod-shaped SIRV2, which are both lytic and infect *Sulfolobus* strains, are the only archaeal viruses whose release mechanism has been studied in detail. Despite the different morphology, both viruses use the same egress strategy, namely viral induced pyramid-like structures on the cell surface, which are unique for archaeal viruses (Prangishvili and Quax, 2011).

In addition to icosahedral viruses, a protein from the hyperthermophilic euryarchaeon *Pyrococcus furiosus* has been reported to form icosahedral particles. Interestingly, this protein has the HK97-like fold (Akita et al., 2007). Similar icosahedral particles have recently been recognized also from the crenarchaeon *Sulfolobus solfataricus* (Heinemann et al., 2011). However, these particles contain no nucleic acids indicating that they are not viruses.

1.4.3. Viruses infecting methanogens

Only five viruses have so far been described for methanogens, and they all belong to the head-tailed virus families *Myoviridae* and *Siphoviridae* (Fig. 4F). In addition, their host range is limited to one order, *Methanobacteriales* (Pina et al., 2011; Prangishvili et al., 2006b). Siphovirus ΨM1 and its more stable deletion derivate ΨM2 from *Methanothermobacter marburgensis* have been studied in more detail (Meile et al., 1989; Pfister et al., 1998). The genome analysis of ΨM2 linear dsDNA revealed for example genes coding for structural proteins and a lytic enzyme degrading host cell wall as well as putative genes functioning in the genome packaging and virion assembly (Pfister et al., 1998). Provirus ΨM100 of *Methanothermobacter wolfeii* shows significant nucleotide sequence similarity to ΨM2 (Luo et al., 2001).

Besides ΨM viruses, myovirus φF1 and siphovirus φF3 infect *Methanothermobacter* species (Nölling et al., 1993). There is also one virus described to infect *Methanobrevibacter smithii* which resembles morphologically ΨM viruses (Prangishvili et al., 2006b). In addition to the virus isolates, one head-tailed VLP designated as *Methanococcus voltae* transfer agent (VTA) has been isolated from a methanogen,

Methanococcus voltae PS (Eiserling et al., 1999).

Spindle-shaped viruses, which are common among archaeal viruses, may also infect methanogens. Thus far, no such virus has been described but a virus-like particle A3 (A3-VLP), which is either spindle shaped or oblate depending on the purification method, has been isolated from *Methanococcus voltae* A3. Like fusello-viruses, A3-VLP contains only one major protein component (Wood et al., 1989). The DNA from A3-VLP has been detected to be integrated in the host chromosome, and A3-VLP could either be a temperate virus or there are defective proviruses in the genome of *Methanococcus voltae* (Krupovič and Bamford, 2008; Wood et al., 1989).

1.4.4. Viruses infecting halophiles

More than half of the isolated archaeal viruses infect halophiles of the phylum *Euryarchaeota* (Atanasova et al., 2012; Pina et al., 2011). Haloarchaeal viruses have been isolated from a variety of sources including salt lakes, salterns, experimental salt ponds, fermented fish sauce, and laboratory cultures of halophilic archaea (Atanasova et al., 2012; Bath et al., 2006; Pauling, 1982; Torsvik and Dundas, 1974; Witte et al., 1997). Viruses infecting halophilic bacteria are also found in hypersaline habitats but less than ten such phages has been described and they all are head-tailed viruses, except for one tailless icosahedral virus (Atanasova et al., 2012; Kukkaro and Bamford, 2009). However, since archaea dominate in hypersaline environments, most likely their viruses do the same. In a recent study, 49 prokaryotic haloviruses were described from nine different locations, and only four isolates infected bacteria (Atanasova et al., 2012).

1.4.4.1. Isolated viruses versus natural virus diversity

Hypersaline habitats are rich of VLPs, and direct EM analyses have revealed concentrations as high as 10^7-10^9 particles per milliliter (Guixa-Boixareu et al., 1996; Oren et al., 1997; Sime-Ngando et al., 2011). Moreover, the diversity of virion morphotypes in these environments is comparable to that of hyperthermic ones (Sime-Ngando et al., 2011). The most abundant particle types observed in hypersaline waters are spindle shaped and spherical (Guixa-Boixareu et al., 1996; Oren et al., 1997; Sime-Ngando et al., 2011). Head-tailed particles belong to the minority, and they have been reported to represent only 1% of VLPs at high salinity (Sime-Ngando et al., 2011).

In contrast to the EM observations, the majority of the isolated haloarchaeal viruses are head-tailed ones (Atanasova et al., 2012; Pina et al., 2011). The dominance of such isolates was demonstrated in the global search of haloviruses where 63% of the 49 isolates were head-tailed viruses (Atanasova et al., 2012). Other morpho-types described for haloarchaeal viruses are tailless icosahedrally symmetric, spindle shaped and pleomorphic (Fig. 4) (Pina et al., 2011). Linear particles resembling those of crenarchaeal viruses have also been observed in hypersaline environments (Santos et al., 2007; Sime-Ngando et al., 2011) but no such virus has yet been isolated.

Most of the isolated haloarchaeal viruses infect hosts from the genera *Haloarcula*, *Halorubrum* or *Halobacterium* (Atanasova et al., 2012; Prangishvili et al., 2006b). No viruses have been isolated infecting for example *Haloquadratum walsbyi*, one of the dominant haloarchaea in hypersaline environments. However, square-shaped halophilic prokaryotic cells have been reported to be infected at least by spindle-

shaped viruses (Guixa-Boixareu et al., 1996).

It has been proposed that the use of culture collection and laboratory strains in the virus isolation instead of dominant environmental strains has biased the range of the known haloarchaeal viruses (Dyall-Smith et al., 2003; Porter et al., 2007). In the description of the 49 new haloviruses, culture collection strains, however, were hosts to almost as many viruses as the environmental strains isolated from the same locations as the viruses (Atanasova et al., 2012). Thus, the bias is more probably caused by the difficulty to cultivate different haloarchaea, to detect plaque formation and to maintain viruses in laboratory conditions.

The viral diversity in hypersaline waters has also been studied using metagenomic approach (Santos et al., 2007; Santos et al., 2010; Sime-Ngando et al., 2011). These metaviromes show only little sequence similarity to cultured haloviruses but they share similarity with each other, despite distant geographical locations (Santos et al., 2010; Sime-Ngando et al., 2011). Environmental, uncultivated haloviruses have also been sequenced in these metagenomic studies (Santos et al., 2007; Santos et al., 2010).

1.4.4.2. Head-tailed viruses

The genomes and gene products of archaeal viruses usually show little sequence similarity to public databases if closely related viruses are not included. However, haloarchaeal head-tailed viruses share several conserved genes, like those encoding capsid and portal proteins, with corresponding bacterial viruses (Krupovič et al., 2010; Prangishvili et al., 2006b). So far, approximately 40 head-tailed viruses infecting haloarchaea have been isolated (Atanasova et al., 2012; Prangishvili et al., 2006b). Like their bacterial counterparts, archaeal head-tailed viruses have linear dsDNA genomes (Pina et al., 2011; Prangishvili et al., 2006b).

All three types of head-tailed viruses, podo-, sipho- and myoviruses (Fig. 4F), are known to infect haloarchaea. However, the majority are myoviruses in contrast to bacterial head-tailed viruses where siphoviruses are the most common isolates (Ackermann and Prangishvili, 2012; Atanasova et al., 2012). Furthermore, only one archaeal podovirus has so far been isolated and it infects "*Haloarcula sinaiiensis*" (Atanasova et al., 2012).

The first archaeal virus to be described was a myovirus, *Halobacterium salinarum* 1 virus (Hs1), and it was isolated from a haloarchaeal laboratory culture (Torsvik and Dundas, 1974). φH and φCh1 resemble morphologically Hs1, and they have also been isolated from laboratory cultures (Schnabel et al., 1982; Witte et al., 1997). Although φH infects neutrophilic *Halobacterium salinarum* and φCh1 infects alkaliphilic *Natrialba magadii*, the completely and partly sequenced genomes of φCh1 and φH, respectively, share a significant sequence similarity (Klein et al., 2002; Schnabel et al., 1982; Witte et al., 1997). φCh1 is exceptional in such a way that it packages host RNA molecules into mature virions in addition to its DNA genome (Witte et al., 1997).

Myoviruses HF1 and HF2, which have been isolated from the same Australian saltern, provide a good example of recombination in hypersaline waters. HF1 infects cells of *Haloferax*, *Haloarcula*, *Halobacterium*, and *Halorubrum* strains whereas HF2 is restricted to *Halorubrum* cells only (Nuttall and Dyall-Smith, 1993). Despite the different host ranges, their genome sequences are over 90% identical (Nuttall and Dyall-Smith, 1993; Tang et al., 2004). Expect for one change, the differences are clustered at the right arm of the genomes which has been proposed to be

a result of a recent recombination event (Tang et al., 2004). Furthermore, HF1 and HF2 have been noticed to be related to a myovirus, *Haloarcula sinaiiensis* tailed virus 2 (HSTV-2), which was recently isolated from Israel (Atanasova et al., 2012; M. K. Pietilä, P. Laurinmäki, D. A. Russell, C.-C. Ko, D. Jacobs-Sera, S. J. Butcher, D. H. Bamford, and R. W. Hendrix, submitted for publication). The MCP of HSTV-2 shows about 40% identity to HalHV1gp089 protein of HF1 and to HF2p101 and HF2p102 proteins of HF2, which are currently assigned as hypothetical proteins. This identity indicates that these three myoviruses share a recent common ancestor and furthermore shows that haloviruses are able to spread over long distances.

1.4.4.3. Tailless icosahedral viruses

Only two tailless icosahedral viruses, both having an internal lipid membrane, have been described to infect haloarchaea (Atanasova et al., 2012; Jaakkola et al., 2012; Kivelä et al., 2006; Porter et al., 2005). Serpentine Lake *hispanica* virus 1 (SH1) and *Haloarcula hispanica* icosahedral virus 2 (HHIV-2) infect the same host and have similar protein profiles and similar sized linear dsDNA genomes (Jaakkola et al., 2012; Porter et al., 2005). Although SH1 has been isolated from an Australian hypersaline lake and HHIV-2 from an Italian saltern, their genome sequences are almost 60% similar revealing the close relationship of the viruses (Atanasova et al., 2012; Jaakkola et al., 2012; Porter et al., 2005). Thus, in addition to head-tailed viruses, SH1 and HHIV-2 demonstrate that related haloarchaeal viruses can be found in geographically distant locations.

A temperate virus SNJ1, which is induced using a mitomycin-C treatment from the lysogenic host *Natrinema* sp. J7-1, is a spherical virus (Zhang et al., 2012). The SNJ1 virion has been shown to contain lipids, and the virus encodes a putative ATPase similar to ATPases found in viruses belonging to the PRD1-adenovirus-like lineage. Thus, it has been proposed that SNJ1 is a tailless icosahedral virus. Interestingly, the genome sequence of SNJ1 circular dsDNA is identical to the sequence of *Natrinema* plasmid pHH205 showing that this plasmid is in fact the provirus of SNJ1 (Zhang et al., 2012).

1.4.4.4. Spindle-shaped viruses

Spindle-shaped particles are abundant in hypersaline habitats, but so far only one such virus has been described (Bath and Dyall-Smith, 1998; Sime-Ngando et al., 2011). *Haloarcula hispanica* virus 1 (His1) has been isolated from a crystallizer pond of an Australian saltern and it resembles morphologically fuselloviruses (Bath and Dyall-Smith, 1998). However, unlike fuselloviruses His1 has a linear dsDNA genome and encodes a putative, type-B DNA polymerase for protein-primed replication (Bath et al., 2006). For these reasons, His1 has been classified into the floating genus *Salterprovirus* (Bath et al., 2006; King et al., 2012). However, the major structural protein of His1 shares sequence similarity with that of SSV1, the type species of the family *Fuselloviridae*, indicating that His1 and fuselloviruses may be related despite their different genome types (Pietilä et al., 2012).

Haloarcula hispanica virus 2 (His2) has been isolated from an Australian salt lake remote to His1 isolation site (Bath et al., 2006). It infects the same host as His1 and has a linear dsDNA genome of similar size (Bath et al., 2006). At first, His2 was proposed to be spindle shaped and distantly related to His1 (Bath et al., 2006; King et al., 2012). However, the only significant

sequence similarity shared by His1 and His2 is between their putative DNA polymerases (Bath et al., 2006), and recently it has been shown that His2 belongs to a group of pleomorphic viruses (see section 4.).

1.5. Haloarchaeal viruses and salinity

Hypersaline environments seem to form a worldwide virus-host playground as haloarchaeal viruses are able to infect hosts originating from geographically distant locations compared to virus isolation sites and there are examples of related viruses isolated from remote sites (Atanasova et al., 2012; Jaakkola et al., 2012). Thus, it is important to consider what role salinity plays in this system.

1.5.1. Virion stability

Like their hosts cells, many haloarchaeal viruses require high salinity to be viable. For example, myovirus φCh1 needs more than 2 M NaCl in order to be infective (Witte et al., 1997). Tailless icosahedral virus SH1 requires at least 1 M NaCl, and magnesium ions are also essential for its infectivity (Porter et al., 2005). For SH1, it has been shown that low salinity conditions cause the release of the genome and dissociation of several structural proteins resulting in empty lipid-core particles (Kivelä et al., 2006).

Some head-tailed viruses, which are sensitive to NaCl reduction, tolerate the reduction if magnesium ions are present (Nuttall and Dyall-Smith, 1993; Pauling, 1982; Schnabel et al., 1982). For example, φH normally requires at least 3.5 M NaCl, but if magnesium ions are available, it tolerates lower NaCl concentrations (Schnabel et al., 1982). In the presence of magnesium ions, *Halobacterium halobium* (i.e. *salinarum*) virus 1 (Hh-1) is almost independent on NaCl (Pauling, 1982). In addition, 2 M concentration of magnesium is enough to maintain the infectivity of tailless icosahedral virus SH1 in the absence of NaCl (Porter et al., 2005).

Moreover, there are haloarchaeal viruses which tolerate low salinity. When siphovirus φN is incubated in distilled water, more than half of the viruses remain infective (Vogelsang-Wenke and Oesterhelt, 1988). Tailless icosahedral virus HHIV-2 tolerates as low as 0.1 M NaCl concentration in contrast to SH1 (Jaakkola et al., 2012). In addition to icosahedral viruses, spindle-shaped virus His1 tolerates low salinity (Bath and Dyall-Smith, 1998). Interestingly, head-tailed viruses HSTV-2 and *Haloarcula vallismortis* tailed virus 1 (HVTV-1) have been shown to be uninfective under low salinity conditions but if the salinity is increased, the viruses are activated (M. K. Pietilä, P. Laurinmäki, D. A. Russell, C.-C. Ko, D. Jacobs-Sera, S. J. Butcher, D. H. Bamford, and R. W. Hendrix, submitted for publication). This might provide a protection mechanism for viruses during low salinity periods in nature. All in all, haloarchaeal viruses seem to tolerate larger range of salinity compared to their host cells (Kukkaro and Bamford, 2009).

1.5.2. Viral life cycles

Viruses can outnumber cells even 100-fold in hypersaline ecosystems, and prokaryotic mortality by protozoans is practically absent is some habitats (Guixa-Boixareu et al., 1996; Oren et al., 1997; Santos et al., 2012; Sime-Ngando et al., 2011). Thus, it has been proposed that viruses play a role in controlling haloarchaeal communities for example in the Dead Sea (Oren et al., 1997). However, it has been shown that viral lysis

has no major effect on prokaryotic cell densities at least in salterns. The high viral numbers are proposed to be due to large burst sizes but it may also reflect the abundance of nonlytic viruses with continuous virus production (Guixa-Boixareu et al., 1996).

1.5.2.1. Adsorption

The adsorption of haloarchaeal viruses has been studied only in terms of adsorption rates, and no host receptors or viral proteins binding to these have yet been determined. However, it has been noticed that closely related haloarchaeal viruses may employ different receptor-binding proteins. On one hand, there are closely related head-tailed viruses with different host ranges (Nuttall and Dyall-Smith, 1993; Tang et al., 2004). On the other, it has been proposed that tailless icosahedral viruses SH1 and HHIV-2 use different proteins in the host recognition, although they infect the same host (Jaakkola et al., 2012).

Haloarchaeal viruses of different morphotypes can be divided into four groups based on their adsorption efficiency at different salinities. In group one, adsorption efficiency is independent on the salinity (Daniels and Wais, 1990; Kukkaro and Bamford, 2009; Pauling, 1982). In group two, maximal adsorption efficiency is achieved at certain salinity, and higher or lower concentrations decrease adsorption efficiency (Kukkaro and Bamford, 2009). In group three, increasing salinity enhances adsorption (Kukkaro and Bamford, 2009; Mei et al., 2007). In group four, increasing salinity causes decreasing adsorption efficiency. For example, the adsorption rate of myovirus Hs1 decreases 30-fold when NaCl concentration increases from 3 to 5 M (Torsvik and Dundas, 1980).

When a virus population infecting *Halobacterium salinarum* was studied from a salt pond, it was observed that most of the viruses had low virulence and this was caused by their slower adsorption rates compared to highly virulent viruses (Daniels and Wais, 1998; Wais and Daniels, 1985). The slow adsorption may maximize the probability of viruses to release their genome into host cells instead of adsorption to nonhost material (Daniels and Wais, 1998). In general, however, viruses isolated from hypersaline environments have adsorption rate constants comparable to those of viruses originating from moderate salinities (Kukkaro and Bamford, 2009).

1.5.2.2. Infection cycle

Progeny virions of haloarchaeal head-tailed viruses are released upon a lysis of infected cells (Nuttall and Dyall-Smith, 1993; Schnabel et al., 1982; Witte et al., 1997). Some of these viruses are temperate having a lysogenic phase in addition to a lytic one (Schnabel et al., 1982; Witte et al., 1997). Tailless icosahedral viruses SH1 and HHIV-2 are virulent (Jaakkola et al., 2012; Porter et al., 2005) while SNJ1 is a temperate virus (Zhang et al., 2012). In contrast, the life cycle of spindle-shaped virus His1 resembles those of creanarchaeal fuselloviruses with prolonged virus production without host cell lysis (Pietilä et al., 2012).

Salinity may not be constant in hypersaline habitats. For example, rainfalls might cause dilution. Furthermore, in solar salterns salinity increases from seawater to NaCl saturation levels. These salinity changes can affect the nature of infection cycles of haloarchaeal viruses as has been reported for two head-tailed viruses of *Halobacterium salinarum* (Daniels and Wais, 1990; Torsvik and Dundas, 1980).

Hs1 infection changes from lytic to persistent when salinity increases. The virus is lytic, when NaCl concentration is approximately 4 M or less. At higher salinities, Hs1 establishes a carrier state,

and the lysis of the infected cells is delayed (Torsvik and Dundas, 1980). The same shift from a lytic to persistent infection has also been described for virus S5100 (Daniels and Wais, 1990). Furthermore, when salinity increases, the eclipse period of S5100 becomes almost three times longer, and the latent period extends and the burst size decreases (Daniels and Wais, 1990).

The observations of Hs1 and S5100 infection cycles have led to the proposal that the carrier state protects cells from the viral lysis when salinity is high enough for the optimal growth of haloarchaea. At the same time, this provides storage conditions for the viruses. When salinity is too low to support the optimal growth of the cells, progeny virions are released ensuring the survival of the viruses (Torsvik and Dundas, 1980; Wais and Daniels, 1985). Some viruses and their host cells may even exist in an equilibrium state in hypersaline environments contributed by slow adsorption, long latent periods and delayed cell lysis (Torsvik and Dundas, 1980).

2. AIMS OF THE STUDY

Only a fraction of the structural diversity of viruses has been characterized. Although there is plenty of morphotypes in the virosphere, helically- and icosahedrally-symmetric viruses remain the best characterized isolates. Thus, in order to better understand the evolution of viruses as well as cellular life, we need to expand our knowledge of all types of viruses. Especially, viruses infecting archaea deserve more attention because they have been shown to represent several previously unknown virion morphotypes. Moreover, archaeal viruses are the least studied virus group compared to bacterial and eukaryotic viruses.

To date, most of the peculiar virion morphotypes of archaeal viruses have been described to those infecting hyperthermophiles. Furthermore, there is a bias between cultivated haloarchaeal viruses and the diversity detected in hypersaline habitats. This indicates that only a small portion of different virus types infecting haloarchaea has been isolated. Direct analyses of environmental samples by metagenomics or EM give us important insights into the viral diversity. However, in order to study viruses as a whole, including virus-host dynamics and virion structures as well as to couple genome sequences and virion morphotypes, it is a necessity to isolate and cultivate viruses and their host cells.

In the present study, hypersaline samples collected from a saltern crystallizer were selected for a screen of new haloarchaeal virus isolates. During the course of this study, a novel virus type infecting archaea, designated as pleolipovirus, was discovered, and the analysis was extended from a characterization of one isolate to the comparison of altogether seven viruses. The aim was to study if these viruses form a new viral family and also to see if they are members of a novel viral lineage having counterparts among viruses infecting hosts from other domains of life. The specific aims of this study were:

1. To investigate the life cycle of the pleolipoviruses and to optimize production and purification methods for this new virion type.

2. To identify the structural constituents and to reveal the virion architecture and organization of the pleolipoviruses using biochemical dissociation studies and EM leading to a virion model.

3. To study the relationship between different pleolipovirus isolates and their connection to other viruses.

3. MATERIALS AND METHODS

Viruses and archaeal strains used in the present study are listed in Tables 3 and 4, respectively. Experimental procedures are summarized in Table 5 and are described in detail in the original publications and references therein.

Table 3. Viruses used in this study.

Virus	Origin	Reference
Halorubrum pleomorphic virus 1 (HRPV-1)	Italy, Trapani	I
Halorubrum pleomorphic virus 2 (HRPV-2)	Thailand, Samut Sakhon	Atanasova et al., 2012
Halorubrum pleomorphic virus 3 (HRPV-3)	Israel, Sedom ponds	Atanasova et al., 2012
Halorubrum pleomorphic virus 6 (HRPV-6)	Thailand, Samut Sakhon	III
Halogeometricum pleomorphic virus 1 (HGPV-1)	Spain, Cabo de Gata	Atanasova et al., 2012
Haloarcula hispanica pleomorphic virus 1 (HHPV-1)	Italy, Margherita di Savoia	Roine et al., 2010
Haloarcula hispanica virus 2 (His2)	Australia, Victoria	Bath et al., 2006

Table 4. Archaeal strains used in this study.

Name	Strain	Host for	Reference
Halorubrum sp.	PV6	HRPV-1	I
Halorubrum sp.	SS5-4	HRPV-2	Atanasova et al., 2012
Halorubrum sp.	SP3-3	HRPV-3	Atanasova et al., 2012
Halorubrum sp.	SS7-4	HRPV-6	III
Halogeometricum sp.	CG-9	HGPV-1	Atanasova et al., 2012
Haloarcula hispanica	ATCC 33960	HHPV-1, His2	Juez et al., 1986
Haloarcula quadrata	ATCC 700850		Oren et al., 1999
Haloarcula japonica	TR1		Takashina et al., 1990
Haloarcula marismortui	ATCC 43049		Oren et al., 1990
"Haloarcula sinaiiensis"	ATCC 33800		Javor et al., 1982
Haloarcula vallismortis	ATCC 29715		Gonzalez et al., 1978
Halorubrum coriense	DSM 10284		Oren and Ventosa, 1996
Halorubrum lacusprofundi	DSM 5036		Franzmann et al., 1988
Halorubrum sodomense	DSM 3755		Oren, 1983
Halorubrum sodomense	DSM 1411		Rodriquez-Valera et al., 1983
Halobacterium salinarum	DSM 3754		Ventosa and Oren, 1996
Halobacterium sp.	DSM 3751		Ross and Grant, 1985

Table 5. Summary of methods used in this study.

Method	Used in
Plaque assay	I, II, III
Virus and archaea isolation	I, III
16S rRNA gene sequencing	I, III
Infection cycle analysis	I, III
Growth and purification of virus particles	I, II, III
Purification of viral DNA using phenol extraction	I
Enzymatic digestions of viral DNA	I
Melting curve determination of viral DNA	I
Polymerase chain reaction and molecular cloning techniques	I, III
Viral genome sequencing and annotation	I
DNA and protein concentration measurements	I, II, III
Agarose gel electrophoresis	I
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and tricine-SDS-PAGE	I, II, III
Ethidium bromide, Coomassie blue and Sudan Black B staining of gels for DNA, proteins and lipids, respectively	I, II, III
Glycoprotein staining of gels	II
Deglycosylation of proteins	II
N-terminal amino acid sequencing and mass spectrometry of proteins	I, II, III
Analytical gel filtration and sedimentation	II
Negative staining and EM	I, II, III
Cryo-electron microscopy (cryo-EM) and tomography and image processing	III
Biochemical dissociations of virus particles	II, III
Extraction of viral and cellular lipids and analysis by thin-layer chromatography (TLC) and mass spectrometry	I, II, III

4. RESULTS AND DISCUSSION

4.1. HRPV-1 – ssDNA virus with a novel archaeal virion morphotype (I)

A variety of VLPs have been observed in hypersaline environments (Guixa-Boixareu et al., 1996; Oren et al., 1997; Sime-Ngando et al., 2011). In order to bring these viruses into the limelight, water samples from a crystallizer pond of a saltern located in Italy were screened for halophilic archaea and bacteria, and the isolated strains were subsequently used as hosts in virus search. A virus was isolated infecting *Halorubrum* sp. PV6, which was identified based on 16S rRNA gene sequencing. EM of negatively stained, highly purified virions revealed pleomorphic particles, which did not resemble any of the previously described archaeal viruses (Pina et al., 2011; Prangishvili et al., 2006a), and thus the virus was designated as *Halorubrum* pleomorphic virus 1 (HRPV-1).

Characterization of HRPV-1 showed that it is a nonlytic, lipid-containing virus. In addition to the lipids, the virion is composed of two major structural proteins, VP3 and VP4. The small-sized VP3 (11 kDa) was predicted to contain four transmembrane (TM) domains indicating that it is an integral membrane protein. The large-sized VP4 (65 kDa) was predicted to contain a C-terminal membrane anchor preceded by a coiled-coil domain, and thus it most probably forms the surface extensions observed by EM (see I, Fig. 3). A minor protein, VP8, was also identified and it was predicted to be an ATPase.

Before this study, all known archaeal viruses had a dsDNA genome (Prangishvili et al., 2006b), and one of the most remarkable findings here was the genome type of HRPV-1 as the analyses revealed that this virus has a circular ssDNA genome. Thus, HRPV-1 was the first archaeal virus to be described which has a single-stranded genome and the first haloarchaeal virus with a circular genome. The single-stranded nature of the genome was confirmed using three different approaches: nuclease treatments, melting curve determination, and single-primer annealing followed by enzyme digestion. The genome of HRPV-1 is 7048 nucleotides (nt) long and has a GC content of 54%. The genome was predicted to contain nine open reading frames (ORFs), of which three encoded structural proteins and were designated as genes *3*, *4* and *8*. ORF1 was predicted to encode a rolling-circle replication initiation protein.

The HRPV-1 genome showed sequence similarity both at nucleotide, albeit only over a short stretch, and amino acid level to the partially sequenced *Haloferax* plasmid pHK2 (Holmes et al., 1995). The complete sequence of pHK2 (10795 nt) has subsequently been determined (Roine et al., 2010), and this revealed that HRPV-1 and pHK2 have gene synteny and significant amino acid sequence similarity from HRPV-1 ORF2 to gene *8* (Holmes et al., 1995; Roine et al., 2010). Moreover, ORF1 of pHK2 is also predicted to encode a replication initiation protein (Holmes et al., 1995). Thus, it has been proposed that pHK2 is actually a provirus (Roine et al., 2010).

Besides the similarity with the plasmid pHK2, a gene block of the HRPV-1 genome was related to a region in the genome of haloarchaeal virus His2 and to regions in the genomes of *Haloarcula marismortui* and *Natronomonas pharaonis*. This block extended from HRPV-1 gene *4* to gene *8*. All these similarities indicated that HRPV-1-like viruses or DNA elements are widespread.

4.2. From one isolate to a world-wide distributed virus group (I, II, III)

Viruses resembling HRPV-1 have subsequently been isolated from geographically distant locations (Fig. 5). *Haloarcula hispanica* pleomorphic virus 1 (HHPV-1) has been isolated from another Italian solar saltern, and it has been shown to be highly similar to HRPV-1 except for having a dsDNA genome (Roine et al., 2010). *Halorubrum* pleomorphic viruses 2 and 3 (HRPV-2 and HRPV-3) and *Halogeometricum* pleomorphic virus 1 (HGPV-1) have been isolated in the global search of haloviruses (Atanasova et al., 2012).

In order to further increase the isolate number of pleomorphic viruses infecting haloarchaea, host strains and viruses were screened from salt crystals collected from a solar saltern located in Thailand (III). A virus was found to infect the strain SS7-4, which was identified to be a member of the genus *Halorubrum*. Based on the virion characterization, this virus was designated *Halorubrum* pleomorphic virus 6 (HRPV-6). HRPV-2 (Atanasova et al., 2012) and HRPV-6 (III) have been isolated from the same location but in subsequent years.

His2 has been proposed to be spindle shaped and distantly related to spindle-shaped virus His1, the type species of the genus *Salterprovirus* (Bath et al., 2006; King et al., 2012). The finding that His2 shares gene synteny and amino acid sequence similarity with HRPV-1 (I) led us to investigate His2 in more detail and it proved to belong to the same group as the pleomorphic isolates (III).

Together, these seven viruses form a unique group of archaeal viruses, and on the basis of the virion morphology and structural components (see sections 4.2.4. and 4.2.5.), they are referred to as pleolipoviruses.

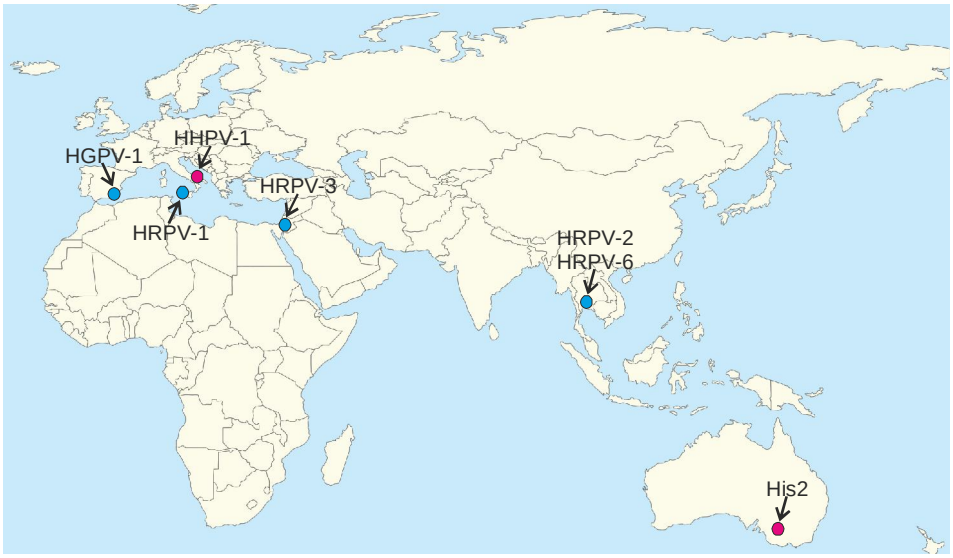

Figure 5. World-wide distribution of pleolipoviruses. Circles indicate virus isolation sites. Blue indicates that the host strain was isolated from the same location as the virus and magenta that the virus was isolated using a culture collection strain (I; III; Atanasova et al., 2012; Bath et al., 2006; and Roine et al., 2010). Source of the map: Wikimedia Commons.

4.2.1. Life cycles

The host range of the pleolipoviruses is extremely narrow as they produced plaques only on the host strains used for their isolation, although representatives from several haloarchaeal genera were tested (I; Atanasova et al., 2012; Bath et al., 2006; Roine et al., 2010). All studied pleolipoviruses formed hazy plaques indicating inhibition of host growth, instead of clear plaques typical of lytic viruses (III).

The life cycle of HRPV-1 was studied in detail by infecting *Halorubrum* sp. PV6 cells in the exponential growth phase (I). The adsorption efficiency of the virus was weak, most likely giving rise to nonsynchronous infection cycles. The virus production started 2-3 h post infection and continued until the cells approached the stationary phase. The virus production reached high levels, up to 10^{11} plaque-forming units (PFU) /ml. Despite this, no cell lysis was observed, and the infection caused only host growth retardation. In addition to continuous virus production, HRPV-1 seemed to persist in infected cells as they produced viruses after several subsequent culturing steps.

HHPV-1 infection affects host cells in the same way as HRPV-1 but results in a slightly higher virus production (Roine et al., 2010). Life-cycle analyses were also performed for HRPV-2, HRPV-3, HRPV-6, HGPV-1, and His2 (III). All viruses resembled HRPV-1 and HHPV-1, although there were minor differences in the host growth retardation and virus production. Previously, it has been reported that His2 might be lytic (Bath et al., 2006), and here some cell lysis was observed during His2 infection, but lysis did not coincide with virus production and was most likely due to stress caused by the continuous virus release.

As a conclusion, the pleolipoviruses induce a rather persistent infection during which progeny virions are produced in a continuous fashion while host growth is retarded. This type of nonlytic infection is common among archaeal viruses (Pina et al., 2011; Porter et al., 2007). For example, spindle-shaped viruses infecting hyperthermophilic archaea are released continuously and virus particles protruding the cell envelope have been observed (Gorlas et al., 2012; Martin et al., 1984; Schleper et al., 1992).

The entry and exit mechanisms of the pleolipoviruses remain still to be determined. The pleomorphic, lipid-containing virion type (see sections 4.2.4. and 4.2.6.) suggests fusion for entry and budding for exit which are typically used by enveloped eukaryotic viruses (Weissenhorn et al., 2007; Welsch et al., 2007). Some eukaryotic viruses utilize endosomal sorting complex required for transport (ESCRT) pathways when budding (Welsch et al., 2007), and the finding of ESCRT homologues in crenarchaea indicates that archaeal viruses may also recruit ESCRT-like cellular components for the budding process (Pina et al., 2011; Samson et al., 2008). However, no budding mechanism has yet been described for any archaeal virus. Furthermore, it is obscure how the virions pass the proteinaceous S-layer of the archaeal host cells (Albers and Meyer, 2011). If some information is obtained about these viral entry and exit pathways, it could also be used to study general mechanisms of membrane-vesicle formation and release as well as membrane fusion in archaeal cells.

4.2.2. Virion infectivity

Some haloarchaeal viruses are extremely sensitive to lowered ionic strength while others tolerate considerably low salinity (Jaakkola et al., 2012; Kukkaro and Bamford, 2009; Porter et al., 2005; Witte et al., 1997; Vogelsang-Wenke and Oesterhelt, 1988). The stocks of the pleolipoviruses contained various salts, and the salt dependence of the viruses was studied. All viruses were sensitive to lowered ionic strength, and HHPV-1 and His2 required the highest salinity to be infective (II, III). For HRPV-1, the effects of different salts including NaCl, KCl, $MgCl_2$, and $CaCl_2$ were studied separately, and the most vital one was NaCl (II). Based on these results, optimal salt buffers were developed for each virus (II, III).

In addition, HRPV-1 infectivity in different pH and temperature conditions was studied, and the results showed that low pH and high temperature inactivated the virus (II). Interestingly, the same has been described for another haloarchaeal virus SH1, which is a tailless icosahedral virus (Porter et al., 2005).

4.2.3. Production and purification of virus particles

High titers (10^9 to 10^{12} PFU/ml) were obtained and the pleolipovirus stocks were stable for several months (I, II, III). For HRPV-1, a liquid culture propagation method was developed and optimized on the basis of the life-cycle studies (I). The liquid culture propagation method for HHPV-1 has also been developed (Roine et al., 2010). The other viruses were purified using the virus stocks (III).

A purification protocol was optimized for HRPV-1 (I), and the same procedure was adjusted for all pleolipoviruses (III). For HRPV-1 and HRPV-3, pre-precipitation cut with polyethylene glycol (PEG) was employed to remove filamentous impurities from the starting material. Subsequently, the virus particles were precipitated using a higher PEG concentration. The concentrated virus particles were purified in a sucrose gradient by a rate zonal centrifugation method yielding "1× purified" virions. Further purification was done in a CsCl gradient by equilibrium centrifugation yielding "2× purified" virions, and the particles were concentrated by differential centrifugation. For the "2× purified" virions, the recovery was approximately 20% of infectious virions, with a specific infectivity of 3×10^{13} PFU / mg of protein (III).

The density of the virions in CsCl was determined (I, III), except for the density of His2 and HHPV-1 has been determined elsewhere (Bath et al., 2006; Roine et al., 2010). All pleolipoviruses have a low buoyant density, from 1.26 to 1.34 g/ml, suggesting the presence of lipids.

4.2.4. Morphology

When negatively stained with ammonium molybdate (AM) or uranyl acetate (UA) and examined in a transmission electron microscope, all studied viruses were pleomorphic varying from spherical to elongated (I, II, III; Atanasova et al., 2012; Roine et al., 2010). However, AM and UA staining resulted in dissimilar looking particles. The stains also decreased the infectivity of the viruses, UA more than AM (III).

Negative-staining EM suggested that the pleolipoviruses have a flexible virion structure not defined by a rigid protein capsid. However, it is also important to emphasize that pleomorphicity has been observed among several archaeal viruses.

For example, spindle- and ovoid-shaped viruses have been reported to be pleomorphic to some extent (Mochizuki et al., 2011; Redder et al., 2009). This illustrates the sensitivity of virion morphology to negative stains and more importantly the plasticity of certain virion types.

Thus, it was essential to study the morphology of the pleolipoviruses in their native hydrated state and to employ cryo-EM. This technique revealed that all seven viruses were roughly spherical and the virion surface was decorated with spike-like protrusions. Noticeably, His2 was not spindle shaped but resembled the other pleolipoviruses. The virion size of the pleolipoviruses varied from 41.1 ± 2.2 nm (HRPV-1) to 70.6 ± 3.6 nm (His2) (Table 6). Some heterogeneity in the genome packaging densities was observed and it may reflect the proposed budding-based assembly of these viruses (III).

Table 6. Summary of pleolipoviruses.

Virus	Host	Genome[a,b]	Genome accession number	Virion size (nm)[c]	Identified structural proteins[b]
HRPV-1	*Halorubrum* sp. PV6	Circular ssDNA (7048)	FJ685651	41.1 ± 2.2	VP3, VP4, VP8
HRPV-2	*Halorubrum* sp. SS5-4	Circular ssDNA (10656)	JN882264	54.0 ± 4.3	VP4, VP5
HRPV-3	*Halorubrum* sp. SP3-3	Circular dsDNA (8770)	JN882265	67.2 ± 5.2	VP1, VP2
HRPV-6	*Halorubrum* sp. SS7-4	Circular ssDNA (8549)	JN882266	48.5 ± 2.7	VP4, VP5
HGPV-1	*Halogeometricum* sp. CG-9	Circular dsDNA (9694)	JN882267	55.5 ± 5.2	VP2, VP3, VP4
HHPV-1	*Haloarcula hispanica*	Circular dsDNA (8082)	GU321093	51.7 ± 4.0	VP3, VP4
His2	*Haloarcula hispanica*	Linear dsDNA (16067)	AF191797	70.6 ± 3.6	VP27, VP28, VP29, VP32

[a] The genome size as nucleotides or basepairs is given in parenthesis.
[b] I; III; Roine et al., 2010; and Senčilo et al., 2012. The proteins identified in I and III are shown in bold.
[c] III.

4.2.5. Structural proteins

In addition to the morphotype, all seven pleolipoviruses have highly similar protein profiles (Fig. 6A). The structural proteins identified here and elsewhere are summarized in Table 6, and they have been shown to share sequence similarity (Senčilo et al., 2012). Five of the viruses have one small-sized and one large-sized major structural protein designated internal membrane and spike proteins, respectively (see sections 4.3.1. and 4.3.2.). HGPV-1 with two internal membrane proteins and His2 with two spike proteins were exceptions to this rule (III).

Glycoprotein staining indicated that the spike protein of HRPV-1, VP4, is glycosylated, and this was confirmed by nonselective deglycosylation, which removes both N- and O-linked oligosaccharide motifs (II). The major glycan of VP4 has now been identified to be an N-linked pentasaccharide attached to at least

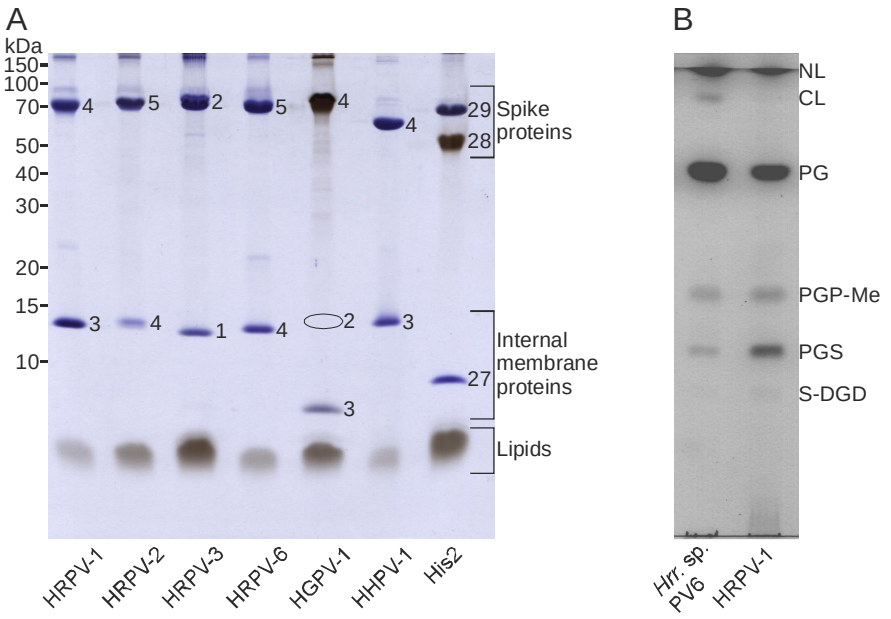

Figure 6. Protein and lipid components of pleolipoviruses. (A) Protein and lipid profiles of highly purified virus particles in a tricine-SDS-PAGE gel stained with Coomassie blue (for proteins) and Sudan Black B (for lipids). Numbers on the gel indicate virion proteins and numbers on the left molecular mass markers. The position of HGPV-1 VP2 protein, which does not appear in a gel of intact virions, is marked by an oval (III). (B) Lipids of *Halorubrum* sp. PV6 and HRPV-1 analysed by TLC. The abbreviations: NL, neutral lipids; CL, cardiolipin; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate; S-DGD, sulfated diglycosylglycerol diether (II).

two sites (Kandiba et al., 2012). The glycosylation was shown to be important for the virus infectivity, and it is proposed to stabilize the protein in hypersaline environments. Glycoproteins have also been detected in other archaeal viruses including bacilliform, rod-shaped, and tailless icosahedral viruses, but the glycosylation of these proteins has not been studied in more detail (Maaty et al., 2006; Mochizuki et al., 2010; Vestergaard et al., 2005). Although the spike protein of HRPV-1 is glycosylated, no gene predicted to encode a glycosyltranferase has been recognized in the HRPV-1 genome (I). Consequently, the virus most likely uses its host glycosylation machinery and therefore this virus system could be utilized to study glycosylation processes in haloarchaeal cells.

Another modification of the spike proteins was observed when VP28 of His2 and VP4 of HGPV-1 were discovered to contain lipid moieties (III). Lipid modifications ranging from the attachment of fatty acids to the addition of isoprenoid lipids have been predicted for or observed in several archaeal proteins (Eichler and Adams, 2005). The lipid moiety and attachment site of the His2 and HGPV-1 spike proteins remain to be identified but no evidence was obtained supporting N-terminal modification. More likely, the lipid modification contributes to the C-terminal membrane anchoring of these spike proteins (see section 4.3.1.) as has been reported for the S-layer glycoprotein of *Halobacterium salinarum*, which contains a covalently attached phospholipid in the C-terminal region (Kikuchi et al., 1999).

4.2.6. Lipids

Initial analysis of HRPV-1 showed that the virion contains lipids (I). Thus, the lipid extracts of HRPV-1 and its *Halorubrum* sp. PV6 host cells were studied by TLC (Fig. 6B) and the lipids were identified by mass spectrometry (II). Both contained three major phospholipids, PG, PGP-Me and PGS. In addition, one minor phospholipid, cardiolipin, and one minor glycolipid, sulfated diglycosylglycerol diether (S-DGD), were identified in HRPV-1 and its host cells. In addition, quantitative analysis of phospholipids revealed that the proportions of different species are similar in the virus and host indicating that HRPV-1 acquires its lipids rather unselectively from the host cell membrane. Furthermore, the neutral lipid composition of HRPV-1 is similar to that of the host cells.

It has been shown that HHPV-1 contains the same phospholipid species as HRPV-1 and acquires lipids unselectively (Roine et al., 2010). Furthermore, TLC analysis confirmed that HRPV-2, HRPV-3, HRPV-6, HGPV-1, and His2 have similar polar lipid profiles as their host cells (III). This indicates that all pleolipoviruses share the unselective lipid acquisition. However, unlike other pleolipoviruses, HGPV-1 has only two major phospholipid species, PG and PGP-Me (III), which is due to the lack of PGS in its *Halogeometricum* host cells (Cui et al., 2010; Montalvo-Rodríguez et al., 1998).

Unlike the pleolipoviruses, prokaryotic tailless icosahedral viruses, which have either an external or internal membrane, acquire lipids selectively from the host lipid pool (Bamford et al., 2005; Laurinavičius et al., 2004a; Laurinavičius et al., 2004b; Maaty et al., 2006; Zhang et al., 2012). This selectivity issue most likely reflects the differences between the assembly pathways of icosahedral and pleomorphic viruses. On one hand, the geometrical constrains associated with the icosahedral capsids are thought to drive the selective lipid acquisition (Laurinavičius et al., 2004a; Laurinavičius et al., 2004b). On the other hand, the nonselective lipid acquisition of the pleolipoviruses further supports budding as their exit mechanism.

4.3. Virion architecture of pleolipoviruses (II, III)

About 30 high-resolution protein structures of archaeal viruses have been solved, and they all come from crenarchaeal viruses. Furthermore, only five of these represent major structural proteins (Krupovič et al., 2012). Besides, only a small number of high-resolution archaeal virion structures has been determined (Pina et al., 2011). This limited information available on archaeal viral structures illustrates the complexity of virus propagation and moreover the asymmetric nature of many archaeal virion morphotypes. Here, the aim was to quantitatively dissociate virions. This would give information on the location and interactions of different components and thus shed light to the virion architecture.

In order to dissociate HRPV-1, a variety of conditions were tested including detergent and protease treatments, acidic conditions, elevated temperature, low salinity, urea, and chloroform (II, III). After each treatment, the dissociation products were analyzed by rate zonal centrifugation. The gradients were fractionated, and the protein, lipid and DNA composition of the fractions was studied. The appearance of dissociation products was also analysed using negative-staining and cryo-EM (II, III). The most close-to-quantitative dissociations revealed the virion architecture, and a virion model was developed (Fig. 7). Three main conclusions were: i) VP4 forms spike structures on the virion surface, ii) VP3 is an

internal membrane protein, and iii) the genome is located inside the vesicle without observed association to any nucleoprotein. Dissociation analyses were also extended to the six other viruses, and the overall virion architecture described for HRPV-1 was amenable for the others as well (III).

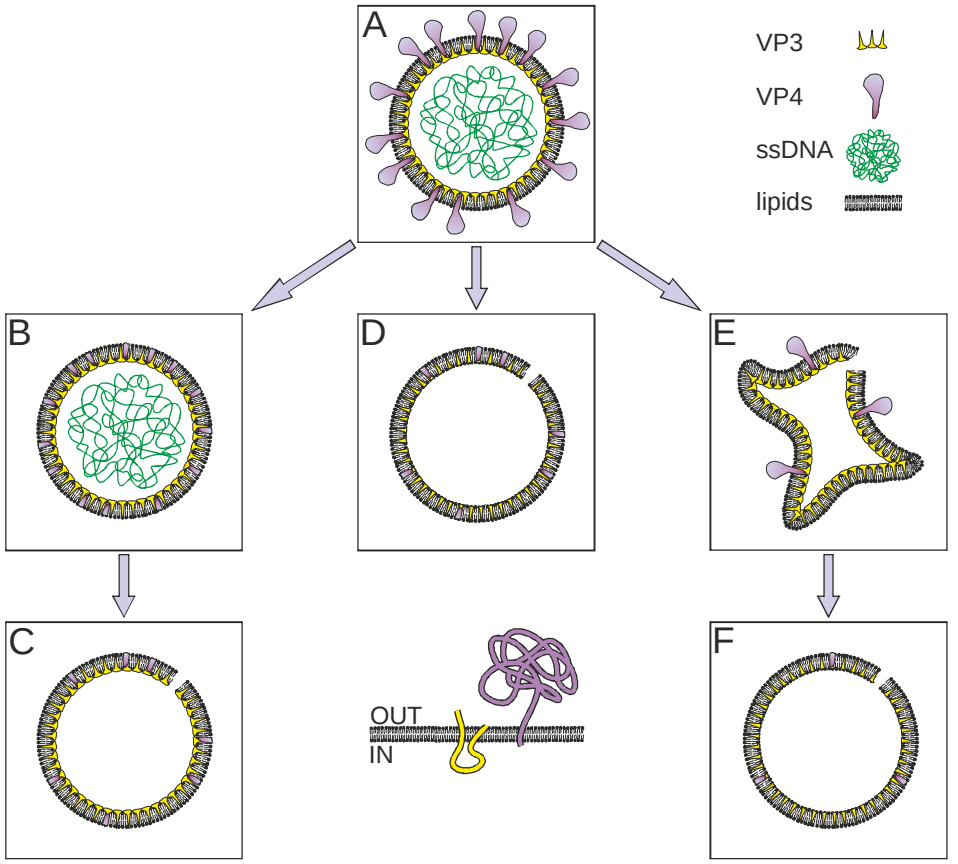

Figure 7. Schematic presentation of the HRPV-1 virion architecture based on the virion dissociation experiments. (A) Infective virion. (B) Virion treated with proteinase K at high salinity resulting in the digestion of the VP4 spikes except for the C-terminal membrane anchor. (C) Proteinase K-treated virion (see panel B) further dissociated at low salinity resulting in the genome release. (D) Virion treated with proteinase K at low salinity resulting in the genome release and partial dissociation of the VP4 spikes. Proteinase K digests VP3 and VP4 except for the membrane-associated domains. (E) Virion treated at low salinity at 60°C resulting in the genome release and dissociation of most of the VP4 spikes. (F) Particle produced at low salinity at 60°C (see panel E) further treated with proteinase K at low salinity resulting in the digestion of VP3 and VP4 except for the membrane-associated domains. The topology of VP3 (yellow) and VP4 (purple) in the membrane is shown between C and F. OUT indicates the outer and IN the inner surface of the virion.

4.3.1. Spike proteins

Proteinase K treatment of HRPV-1 at high salinity resulted in DNA-containing lipid vesicles (Fig. 7B), and EM revealed that the spikes were missing (II, III). The only virion protein digested was VP4, except for that its C-terminal fragment remained associated with the vesicle. This fragment contains the predicted TM domain. Thus, it was concluded that VP4 forms the spike structures and has a C-terminal membrane-anchored domain.

Low-salinity dissociation at 60°C or Nonidet-P40 treatment removed most of the spikes of HRPV-1 (Fig. 7E). The dissociated spikes were studied by analytical gel filtration and sedimentation, but the analyses did not reveal the multimericity of VP4 and only indicated that the dissociated spikes are most likely elongated (II).

The large-sized major structural proteins of the pleolipoviruses have a signal peptide and a predicted C-terminal TM domain (I, III; Bath et al., 2006; Roine et al., 2010; Senčilo et al., 2012). As it was shown for HRPV-1, the protease treatment of the other viruses at high salinity verified that these proteins are located on the virion surface and they were designated as spike proteins. Furthermore, the protease digestion confirmed that they interact with the membrane using their C-terminal region (II, III).

The pleolipoviruses could be divided into three groups based on the protease digestion products of their spike proteins (III). In group one, the digestion produced major soluble fragments. In group two, both major soluble fragments and a membrane-associated domain were observed. In group three, the spike proteins were digested leaving only a membrane-associated domain. These differences emphasize the most likely role of the spike proteins in host cell recognition, which is also supported by the lower sequence identity of the spike proteins compared to the internal membrane proteins (Senčilo et al., 2012).

4.3.2. Internal membrane proteins

Several dissociation conditions of HRPV-1 caused the release of the genome without associated proteins (II). Thus, the HRPV-1 genome resides inside the vesicle without forming a nucleoprotein complex.

Low-salinity dissociation of HRPV-1 at 37°C caused the partial dissociation of the spikes in addition to the genome release (II). However, VP3 remained associated with the empty vesicles. Proteinase K treatment at the same conditions resulted in the digestion of VP3 leaving two membrane-associated fragments, one at the N- and the other at the C-terminus, both containing a predicted TM domain (Fig. 7D). Because only the vesicle opening allowed the digestion of VP3, it was concluded that VP3 faces the virion interior and thus was designated as an internal membrane protein (II).

In order to study VP3 contribution to the membrane layer, different vesicle forms of HRPV-1 were compared using cryo-EM (III). No major differences were observed between the empty lipid vesicles containing VP3 (Fig. 7C) and those having only the two membrane-associated domains of the same protein (Fig. 7D). The virions and empty, VP3-containing lipid vesicles were also subjected to tomographic analysis, and membrane subtomograms were averaged from both particle types. The radial density profile over the averaged membrane revealed a 4.2-nm-thick continuous layer. Thus, VP3 is mostly embedded in the membrane and does not form a thick matrix-type layer on the virion inner surface or an ordered protein capsid.

For the six other viruses, protease digestions and low-salinity dissociations showed that the small major structural proteins are membrane proteins (III). In addition, it could be confirmed that most of them are located on the inner surface of the membrane vesicle and thus played the same role as VP3 of HRPV-1. The topology of HRPV-3 VP1, HRPV-6 VP4 and HGPV-1 VP3 could not be concluded based on the dissociation studies, but because these proteins show sequence similarity to the internal membrane proteins of the other viruses (Senčilo et al., 2012), they were assigned the same function.

Based on the dissociation analyses, no nucleoproteins were identified in any of the pleolipoviruses. However, HRPV-2 and HRPV-6 were the only viruses whose genome was not released by the low-salinity dissociation at 37°C but required a protease addition. This indicates that the internal membrane proteins of HRPV-2 and HRPV-6 may interact more strongly with the genome than do the homologous proteins of the other viruses.

4.3.3. Pleomorphicity

The pleolipoviruses displayed extended light-scattering zones after rate zonal centrifugation indicating that the particles have variable sedimentation coefficients (III). Detailed analysis of HRPV-1 showed that there were no major differences in the composition or specific infectivity of the virions nor did the virion size increase significantly along the light-scattering zone (III). This points to differences in the shape factor of the particles, which is most likely due to the floppy vesicle-like nature of the pleolipoviruses, and consequently reflects their pleomorphic nature (Table 6).

Sedimentation analysis of HRPV-1 particles indicated that the ratio of VP3 and VP4 is approximately the same in all particles (III). However, it is not clear how many copies of VP3 and VP4 are found per particle. Also, possible interactions between the structural proteins remain to be studied.

During dissociation analyses it was observed that the spikeless DNA-containing lipid vesicles of HRPV-1 are more uniform than the virions, indicating that the spikes may contribute to the pleomorphicity (II). Cryo-electron tomography was used to provide more insights into this issue (III). The three-dimensional tomographic reconstruction of the HRPV-1 virions revealed spherical particles covered by spikes. The average structure of the VP4 spikes was determined using the membrane subtomograms of the virions. The iteratively aligned and averaged spike subvolumes revealed a club-shaped structure tapering towards the membrane. When the averaged structure was placed into individual virion tomograms, it was observed that the spikes are randomly distributed on the virion surface instead of forming regular arrays.

The glycoprotein spikes of pleomorphic Uukuniemi virus, which belongs to the family *Bunyaviridae*, are arranged on an icosahedral lattice and most probably determine the structure of the enveloped virion (Överby et al., 2008). It has been proposed that Uukuniemi virus represents an intermediate form between strictly icosahedrally-symmetric and pleomorphic viruses. Thus, as the spikes of HRPV-1 are randomly distributed on the virion surface, the pleolipoviruses can be considered as truly pleomorphic viruses.

4.3.4. Virion organization

The pleolipoviruses clearly differ from other enveloped viruses. First of all, such viruses typically have nucleoproteins associated with the genome. Globuloviruses

provide an example among archaeal viruses as their lipid envelope encloses a helical nucleoprotein complex (Häring et al., 2004). In contrast, the pleolipoviruses have no nucleoprotein, and the genome most likely resides unordered inside the viral vesicle (II, III). Furthermore, enveloped viruses often have a matrix protein on the inner surface of the lipid vesicle. The matrix protein M1 of influenza virus contributes to the virion structure and mediates the interaction between the lipid vesicle and ribonucleoprotein core. Influenza virions contain also an integral membrane protein, M2, which is essential for virion assembly and budding (Rossman and Lamb, 2011). The internal membrane protein VP3 of HRPV-1 does not form a matrix but is mostly embedded in the membrane (II, III). Thus, it may resemble functionally both influenza virus M1 and M2 proteins.

The virion assembly of the pleolipoviruses remains to be studied, but the structural studies provide some clues. The viral proteins are most likely incorporated into the host cell membrane and then the viral genome interacts with these membrane regions and budding occurs. The vesicle size is thus determined by the genome size, and this is indeed seen when the genome and virion sizes of the pleolipoviruses are compared (Table 6). The virion architecture also suggests an important role for the internal membrane proteins in the assembly. Furthermore, these proteins are highly conserved which supports their importance for the assembly (Senčilo et al., 2012).

The spike proteins of the pleolipoviruses most probably function as cell-receptor binding and fusion proteins during the viral entry, like the envelope glycoproteins of eukaryotic viruses (Weissenhorn et al., 2007). However, the spike proteins may also take part in the budding during the exit of progeny virions. Enveloped eukaryotic viruses can be divided into different categories based on whether budding is primarily driven by: i) the inner core proteins, ii) the envelope glycoproteins, or iii) both envelope and inner proteins. If budding is dependent only on the envelope glycoproteins, these proteins usually form an ordered lattice on the virion surface (Welsch et al., 2007). Thus, it seems unlikely that the pleolipoviruses with randomly distributed spikes would recruit the spike proteins alone but may utilize both protein species or only internal proteins in the budding.

4.4. Classification of pleolipoviruses (I, II, III)

The properties of the pleolipoviruses are summarized in Table 6. A comparative genomic study of the pleolipoviruses has shown that these viruses share gene synteny and that there is significant sequence similarity between several proteins or predicted gene products (Senčilo et al., 2012). Thus, both genome sequences and virion architecture (I, II, III) show that the pleolipoviruses are closely related.

The virion architecture of the pleolipoviruses is conserved, but the genome type differs (Table 6) which is most likely due to different replication mechanisms. HRPV-1, HRPV-2, HRPV-6, and HHPV-1 are predicted to use rolling-circle replication (I; Roine et al., 2010; Senčilo et al., 2012). However, only HHPV-1 encloses the double-stranded form (Roine et al., 2010). His2 most likely uses protein-primed replication (Bath et al., 2006), and the replication mechanism of HRPV-3 and HGPV-1 remains unknown (Senčilo et al., 2012). This variety of replication strategies is consistent with the observations that replication-associated genes can be exchanged by HGT (Bamford, 2003; Krupovič and Bamford, 2007).

Already in the earliest hierarchical systems of virus classification, the first criterion was the nucleic acid type of the virus (Lwoff and Tournier, 1966). Today,

the genome type still plays a significant role (King et al., 2012). However, the pleolipoviruses demonstrate how related viruses can have different genome types, either single- or double-stranded, linear or circular DNA (Table 6) and thus challenge the use of the nature of the viral nucleic acids as a main classification criterion, at least within DNA viruses. It has to be mentioned, though, that there have yet been no cases where DNA and RNA viruses have been shown to be closely related.

The genome analysis of the first pleolipovirus isolate, HRPV-1, implied that there is a block of conserved genes defining these viruses (I). Sequencing of the other isolates (Roine et al., 2010; Senčilo et al., 2012) and further characterization of His2 (III) revealed that they all share the same gene block (Fig. 8). This consists of five core genes including two genes encoding the major structural proteins, one encoding a putative ATPase, and two genes with an unknown function (Senčilo et al., 2012). Although HGPV-1 has two genes encoding internal membrane proteins and His2 has two genes for spike proteins, both genes can be considered as core genes because they serve the same function (III). The archaeal spindle-shaped viruses of the family *Fuselloviridae* share 13 core genes, which most likely reflects their larger genome size compared to the pleolipoviruses (Redder et al., 2009; Senčilo et al., 2012).

In addition to the world-wide distribution of the pleolipovirus isolates, putative proviruses have been detected in a variety of haloarchaeal genomes including representatives from the genera *Haloarcula*, *Haloferax*, *Halomicrobium*, *Halopiger*, *Halorhabdus*, *Natrialba*, and *Natronomonas* (I; Dyall-Smith et al., 2011; Roine et al., 2010; Senčilo et al., 2012). In addition, the pHK2 plasmid of *Haloferax lucentense* is most likely a pleolipoprovirus (Roine et al., 2010). Furthermore, a metagenome from a hypersaline lake was shown to contain a sequence similar to the pleolipoviruses (Sime-Ngando et al., 2011).

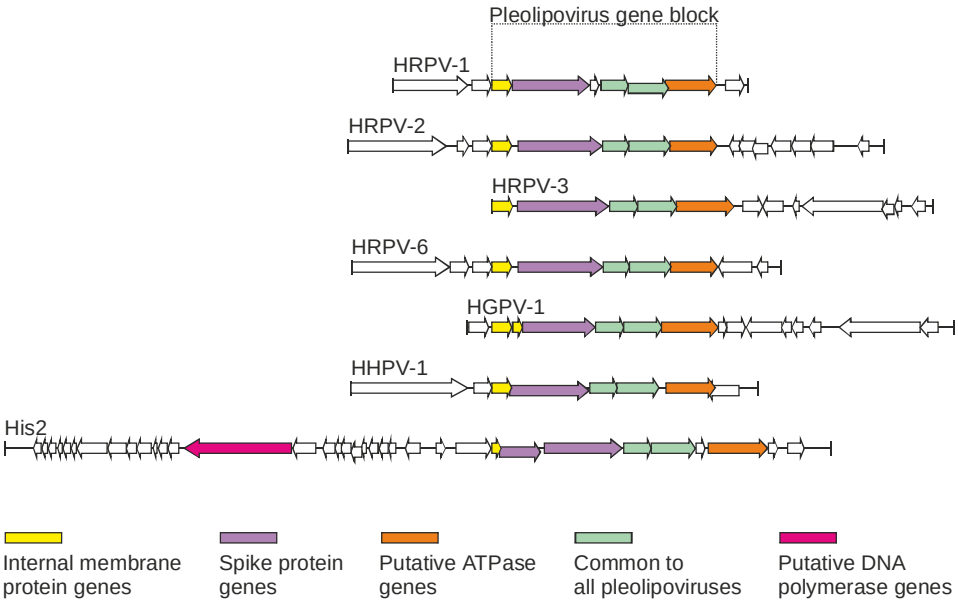

Figure 8. Gene block conserved in the pleolipoviruses. Genes or ORFs found in all pleolipoviruses are indicated by yellow, violet, orange, and green, and the putative DNA polymerase gene of His2 is indicated by magenta (I; Bath et al., 2006; Roine et al., 2010; and Senčilo et al., 2012).

4.4.1. Family *Pleolipoviridae*

Nine families and one unassigned genus containing archaeal viruses can be found in the most recent ICTV report (King et al., 2012). However, several archaeal viruses await official classification, and thus the known archaeal viruses could be classified into about 15 families or corresponding groups (Ackermann and Prangishvili, 2012). The discovery of the pleolipoviruses led us to propose a new archaeal viral family, *Pleolipoviridae* (from the Greek pleo, for more or many; from the Greek lipos, for lipid).

The dissociation results showed that although the pleolipoviruses share the same simple virion architecture, there are subtle differences. Based on the structural information, the following subgroups were formed: i) HRPV-1, HHPV-1 and His2, ii) HRPV-2 and HRPV-6, and iii) HRPV-3 and HGPV-1 (III). In contrast, the genomic data led to different subgroups: I) HRPV-1, HRPV-2, HRPV-6, and HHPV-1, II) HRPV-3 and HGPV-1, and III) His2 (Senčilo et al., 2012). These groups were formed based on the sequence similarity and the presence of a putative replication initiation protein (group I), transcriptional regulator (group II) or DNA polymerase (group III). Thus, although HRPV-3 and HGPV-1 seem to form a separate group according to both structural and genomic data, the isolate number is currently too low to form distinct genera, and only one genus is proposed: *Pleolipovirus* with HRPV-1 as a type species.

Previously, it has been proposed that His2 belongs to the genus *Salterprovirus* of spindle-shaped viruses (Bath et al., 2006). However, cryo-EM showed that His2 is not a spindle-shaped virus but belongs to the family *Pleolipoviridae*, and the analysis of the structural components confirmed this. Furthermore, it was recently shown that His1 has only one major structural protein and no free lipids in contrast to His2 with three major structural proteins and a lipid membrane (Pietilä et al., 2012). Thus, His1 and His2 are not related. However, their genomes might have regions originating from the same source. The His2 genome is composed of two parts transcribed into opposite directions (Bath et al., 2006). The first part, which contains the putative DNA-polymerase gene, is responsible for replication and the other part, which contains the pleolipovirus-gene block, is responsible for virion assembly and architecture. Thus, His2 might have originated by a recombination event between His1- and pleolipovirus-like ancestors.

4.4.2. New lineage of viruses with a membrane envelope

The conserved virion architecture of the pleolipoviruses indicates that they have a common origin (III). More importantly, similar morphology has been observed among bacterial mycoplasmaviruses. L172 is a pleomorphic phage infecting *Acholeplasma laidlawii*, and it has a circular ssDNA genome of 14 kb. Interestingly, there are only two major protein components in L172 virions and their size is close to those of the pleolipoviruses (Dybvig et al., 1985). Furthermore, L172 is a lipid-containing virus and seems to acquire phospholipids unselectively from the host cell membrane (Al-Shammari and Smith, 1981). Thus, the similarities between the archaeal pleolipoviruses and bacterial mycoplasmavirus imply that these viruses could form a viral lineage with an ancient origin (I, II, III). The pleolipovirus-like lineage would be the first one containing enveloped viruses without a nucleocapsid. However, until such a lineage can be established more information is needed from the L172 virion and high resolution protein or virion structures of both archaeal and bacterial viruses need to be solved.

Currently, all four established viral lineages contain icosahedral viruses only. Other lineages have been proposed but especially enveloped viruses have proved to be a challenge (Abrescia et al., 2012; Abrescia et al., 2010). In particular, it has been difficult to determine the viral self of these viruses. The envelope proteins are most often involved in the interaction with host cells and thus cannot be considered as the viral self. Furthermore, the envelope proteins, which function in membrane fusion, are not suitable for structure comparisons due to their flexible nature (Abrescia et al., 2012; Bamford, 2003; Bamford et al., 2002). Thus, inner structural proteins like matrix and nucleoproteins may be better candidates for the viral self (Abrescia et al., 2012; Abrescia et al., 2010). Based on this, the spike proteins of the pleolipoviruses are not considered to represent the viral self. Therefore, the internal membrane proteins seem to be a more plausible choice for the viral self because they are most probably essential for the virion assembly (III).

5. CONCLUSIONS AND FUTURE PROSPECTS

In this thesis, a novel group of seven pleomorphic, lipid-containing viruses designated as pleolipoviruses was described. HRPV-1 is the first isolated ssDNA virus infecting archaea, and this finding showed that archaeal viruses are not restricted to dsDNA genomes. The pleolipoviruses have a simple virion architecture with two major structural protein species incorporated into the lipid vesicle. The spike proteins decorate the virion outer surface and are proposed to function in host recognition and membrane fusion during the entry. Three pleolipoviruses have lipid- or glyco-modified spikes. The internal membrane proteins are embedded in the viral membrane and are most likely essential for the virion assembly. The genome resides inside the vesicle without forming a nucleoprotein complex. The lack of matrix and nucleoproteins differentiate the pleolipoviruses from the other enveloped viruses.

In addition to the morphotype and virion structural components, the pleolipoviruses share a similar life cycle resulting in high virus production which retards host cell growth. Budding is the most likely exit mechanism as the pleolipoviruses acquire their lipids unselectively from the host lipid pool. The genomic comparison published elsewhere shows that the pleolipoviruses have a collinear gene organization and share significant sequence similarity at the amino acid level (Senčilo et al., 2012). Thus, the pleolipoviruses are closely related and a new viral family, *Pleolipoviridae*, has been proposed. Furthermore, the archaeal pleolipoviruses share similarities with a pleomorphic bacterial virus, and these viruses may form a viral lineage.

The discovery of a new archaeal virus group opened a whole new research field among these extremophilic viruses, and although much has been learned about the virion architecture and genomes of the pleolipoviruses, several aspects await to be studied. The high-resolution structures of the spike and internal membrane proteins would be highly informative and allow a better comparison of the pleolipoviruses. The structures would also bring light on the functions of these proteins. Also, the fusion activity of the spike proteins remains to be shown. Besides the entry, the exit mechanism of the pleolipoviruses, and especially how the virions penetrate the cell envelope without killing the cell, needs to be analysed. Finally, the assembly pathway of these viruses could be put together if the above mentioned pieces of data can be resolved. In addition, the study should also be extended to the bacterial members of the proposed pleolipovirus-like lineage in order to establish their ancient common origin.

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