PROKARYOTIC MICROORGANISMS, VIRUSES, AND ANTIMICROBIAL AGENTS FROM HYPERSALINE ENVIRONMENTS

Nina S. Atanasova

Institute of Biotechnology and
Division of General Microbiology
Department of Biosciences
Faculty of Biological and Environmental Sciences and
Viikki Doctoral Programme in Molecular Biosciences
University of Helsinki

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, for public examination in the auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on 26th of April 2013, at 12 o'clock noon.

Helsinki 2013
To my family
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


II Atanasova, N.S., Pietilä, M.K., and Oksanen, H.M. Diverse antimicrobial interactions of halophilic archaea and bacteria extend over geographical distances and cross the domain barrier. Microbiology Open (under consideration)


The publications are referred to in the text by their roman numerals.
ABBREVIATIONS

ARMAN archaeal Richmond Mine acidophilic nanoorganisms
CRISPR clustered regularly interspaced short palindromic repeats
cryo-EM electron cryo-microscopy
cryo-TM electron cryo-tomography
DNA deoxyribonucleic acid
ds double stranded
EM electron microscopy
ICTV International Committee on Taxonomy of Viruses
MCP major capsid protein
nt nucleotide
ORF open reading frame
PFU plaque-forming unit
PG phosphatidyl glycerol
PGP-Me phosphatidyl glycerophosphate methyl ester
PGS phosphatidyl glycerosulphate
RNA ribonucleic acid
rRNA ribosomal RNA
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SHOW square haloarchaea of Walsby
S-layer surface layer
ss single stranded
SSU small subunit
Tat twin-arginine translocation
TEM transmission electron microscopy
TGD triglycosyl glycerodiether
TLC thin-layer chromatography
tRNA transfer RNA
UV ultra violet
VLP virus-like particle
VP virion protein
YNP Yellow Stone National Park
ABBREVIATED VIRUS NAMES

ABV    Acidianus bottle-shaped virus
ACV    Aeropyrum coil-shaped virus
AFV    Acidianus filamentous virus
APOV1  Aeropyrum pernix ovoid-shaped virus 1
APBV1  Aeropyrum pernix bacilliform virus 1
APSV1  Aeropyrum pernix spindle-shaped virus 1
ASV1   Acidianus spindle-shaped virus 1
ATV    Acidianus two-tailed virus
HATV-1 Haloarcula head-tailed virus 1
HATV-2 Haloarcula head-tailed virus 2
HCTV-1 “Haloarcula californiae” head-tailed virus 1
HCTV-2 “Haloarcula californiae” head-tailed virus 2
HCTV-5 “Haloarcula californiae” head-tailed virus 5
HGPV-1 Halogeometricum pleomorphic virus 1
HGTV-1 Halorugnum head-tailed virus 1
HHIV-2 Haloarcula hispanica icosahedral virus 1
HHPV-1 Haloarcula hispanica pleomorphic virus 1
HHTV-1 Haloarcula hispanica head-tailed virus 1
HHTV-2 Haloarcula hispanica head-tailed virus 2
HJTV-1 Haloarcula japonica head-tailed virus 1
HJTV-2 Haloarcula japonica head-tailed 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
HRTV-1 Halorubrum head-tailed virus 1
HRTV-2 Halorubrum head-tailed virus 2
HRTV-3 Halorubrum head-tailed virus 3
HRTV-4 Halorubrum head-tailed virus 4
HRTV-5 Halorubrum head-tailed virus 5
HRTV-6 Halorubrum head-tailed virus 6
HRTV-7 Halorubrum head-tailed virus 7
HRTV-8 Halorubrum head-tailed virus 8
HRTV-9 Halorubrum head-tailed virus 9
HRTV-10 Halorubrum head-tailed virus 10
HRTV-11  *Halorubrum* head-tailed virus 11
HRTV-12  *Halorubrum* head-tailed virus 12
HSTV-1  "Haloarcula sinaiiensis" head-tailed virus 1
HSTV-2  *Halorubrum sodomense* head-tailed virus 2
HSTV-3  *Halorubrum sodomense* head-tailed virus 3
HTV-1  Halophilic head-tailed virus 1
HVTV-1  *Haloarcula vallismortis* head-tailed virus 1
HVTV-2  *Haloarcula vallismortis* head-tailed virus 2
PAV1  *Pyrococcus abyssi* virus 1
PBCV-1  *Paramecium bursaria* Chlorella virus 1
PSV1  *Pyrobaculum* spherical virus 1
SCTP-1  *Salicola* head-tailed phage 1
SCTP-2  *Salicola* head-tailed phage 2
SCTP-3  *Salicola* head-tailed phage 3
SIFV  *Sulfolobus islandicus* filamentous virus
SIRV1  *Sulfolobus islandicus* rod-shaped virus 1
SIRV2  *Sulfolobus islandicus* rod-shaped virus 2
SNDV  *Sulfolobus neozealandicus* droplet-shaped virus
SRV  *Stygiolobus* rod-shaped virus
SSIP-1  *Salisaeta* icosahedral virus 1
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
TPV1  *Thermococcus prieurii* virus 1
TTSV-1  *Thermoproteus tenax* spherical virus 1
TIV1  *Thermoproteus tenax* virus 1
SUMMARY

Archaea are commonly found in the most extreme environments on Earth, such as geothermal hot springs, submarine hydrothermal vents and hypersaline lakes. They are also an important part of the biosphere in soils and different types of aquatic environments. Hypersaline environments in different parts of the world are dominated by extremely halophilic archaea from the family Halobacteriaceae, but contain also halophilic bacteria and the green algae Dunaliella, which is the sole primary producer in such ecosystems. Since cellular predators are nearly absent in extreme salinities, haloviruses are the main force influencing cell diversity and evolution of halophilic microorganisms. From the studied aquatic environments, hypersaline lakes and salterns contain the highest numbers of virus-like particles. A high percentage of these are considered to represent archaeal viruses. To date, only a handful of archaeal viruses have been characterized. The described ones infect extreme halophiles, hyperthermophiles, or methanogens and are most famous for having either unique virion architectures, or morphotypes resembling viruses that infect members of the other two domains.

The number of viruses on the biosphere is astronomical and viruses represent the greatest genetic diversity on the Earth. However, the limited protein fold space restricts the ways viral capsids can be assembled and it has been proposed that the vast number of viruses can be organized into a limited amount of distinct morphotypes. In order to test this hypothesis, a global sampling of nine different hypersaline environments was performed. Close to 100 strains of halophilic archaea and bacteria and 49 viruses infecting them were isolated. These viruses approximately doubled the number of known archaeal viruses. The majority of the viral isolates were head-tailed myoviruses and siphoviruses and for the first time, podovirus morphology was observed for an archaeal virus. From the four isolated halophilic bacteriophages, three were head-tailed and one, designated as SSIP-1, was the first tails icosahedral membrane-containing phage described for a halophilic bacterium. An archaeal virus with a similar morphology was also isolated. It is probable, that these two viruses belong to the structure-based lineage of PRD1-like viruses, which are considered to have a common ancestor. In addition to these well-known virus morphotypes, we isolated a group of viruses with a novel, pleomorphic morphology. The isolated four viruses were compared with the previously described pleomorphic HRPV-1, HHPV-1 and His2. These seven viruses,
designated as pleolipoviruses, had similar virion architecture consisting of a membrane vesicle which encloses the ssDNA or dsDNA genome without associated nucleoproteins. Pleolipoviruses form a group of related viruses despite having different genome types and thus challenge the current classification of viruses based on similar genome types and replication mechanisms.

In addition to virus morphology, we studied microbial interactions in nine hypersaline environments. One third of the isolated archaea and bacteria were found to produce antimicrobial agents called halocins. Several of the identified halocin producers were inhibiting a broad range of sensitive strains, even those belonging to a different domain of life. When virus-host interactions were studied, numerous viruses were found to infect hosts from geographically distant locations. The same observation was done for halocin production-sensitivity interactions, indicating that halophilic microorganisms are globally distributed and able to produce broad spectrum antibiotics.
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2. AIMS OF THE STUDY 

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1 INTRODUCTION

![Schematic representation of the three domains of life. Modified from Woese et al., 1990 and Madigan and Martinko, 2012.](image)

1.1 Extreme environments and their microbiota

The diversity of different life forms on our planet is enormous, ranging from simple parasites to multicellular organisms. Since most areas on the Earth surface support life, some difficulties might arise in defining what is “extreme” or “normal” in terms of life conditions, environments, and survival. In general, extreme environments are defined as those with low species diversity and which lack whole taxonomic groups of organisms (Brock, 1979, reviewed in Ventosa, 2006). The English word extreme comes from the Latin extrēmus, which means uttermost, utmost, or last (Morwood, 2012). This word in itself describes the nature of extreme environments where life conditions can be described as being somewhat “on the edge”. The major requirement for life to exist is the availability of liquid water within the cell, because all enzymatic reactions, proteins and nucleic acids require an aquatic environment for functioning. These needs are well met in an environment with a temperature around 20-30 °C, a pressure of 1 ATM, neutral pH, and water available (Rothschild and Mancinelli, 2001; Satyanarayana et al., 2005). However, when subjected to extreme parameters, such as elevated or lowered temperatures, high alkalinity or acidity, high or low pressure, high salinity, low water-level activity, or intense ultra violet (UV) radiation, these essential functions become seriously compromised.
However, the tremendous capacity of life is known to cover even the extreme
niches on the Earth. These special kinds of environments are inhabited by
organisms called extremophiles, which possess a cell structure and metabolism
well adapted to extreme conditions (Rainey and Oren, 2006; Pikuta et al., 2007).
Most extremophiles are microorganisms, although some multicellular
representatives are known too, especially in the cold (psychrophilic)
environments. In addition, some mesophilic organisms have various developing,
resting or dormant stages during which they can withstand extreme conditions
(Rothschild and Mancinelli, 2001; Tynan et al., 2010).

Since 1990, cellular organisms have been classified into the three domains of
life, Archaea, Bacteria and Eucarya based on phylogenetic analyses of small
subunit (SSU) ribosomal RNA genes (Fig. 1.) (Woese et al., 1990). All the three
domains contain extremophiles, although in the most hostile environments, the
predominance of archaea seems evident (Rainey and Oren, 2006). Ever since
their discovery, archaea have been considered as the most supreme
extremophiles, but lately their abundance has been noticed also in mesophilic
aquatic and terrestrial environments (DeLong, 1992; Abreu et al., 2001;
Leininger et al., 2006; Brochier-Armanet et al., 2008). Simultaneously, bacteria
and eukaryotes have been described from extreme environments (Rothschild
and Mancinelli, 2001). During the last century, the methods to culture and study
extremophiles have improved tremendously and various extreme niches on our
planet are now well known to be flourishing with life (Rainey and Oren, 2006).

The temperature specific extreme environments include psychrophilic and
hyperthermophilic environments. Psychrophilic environments can be found at
the alpine and polar regions of the planet and in the deep levels of the oceans.
Permafrost, snow, ice, and cold water together actually constitute more than half
of the Earth’s surface (Cavicchioli, 2006). In sea ice, there are channels within
the ice crystals, which are able to hold liquid water and nutrients even at
temperatures close to -40 °C. Psychrophiles are commonly found in this
interface between solid ice and fluid (Dieckmann and Hellmer, 2010). The
abundance of unsaturated lipids in the cell membranes and the synthesis of anti-
freezing agents are common ways of adjustment for psychrophiles (Cavicchioli,
2006).

The other temperature extreme, hyperthermophilic environments, are
mainly located at sites of tectonic plate convergence and thus exhibit volcanic or
geothermal activity (Stetter, 2002). Terrestrial hyperthermophilic environments
include volcanos and hot springs, whilst hydrothermal vents and black smokers
are typical submarine hyperthermophilic environments. The latter are located in
ocean floors and are also subjected to extremely high pressure, which can raise
the temperature up to 400 °C (Stetter, 2002). Both extremes of pH are also
characteristics of hyperthermophilic environments, as they can be highly acidic
due to microbial oxidation of reduced sulfur compounds (Stetter, 2006) or
alkaline due to carbonates, chlorides and silicates (Kevbrin et al., 2003; Grant,
2006). The hottest environments on Earth contain solely prokaryotic
microorganisms called hyperthermophiles, which possess certain special
features such as thermostable proteins with increased hydrogen bonding,
reverse gyrase enzyme, and tetraether membrane lipids, which all in part
contribute to the ability of these organisms to thrive in these conditions
(Forterre, 2002).

As the above description illustrates, extreme environments are often
characterized by more than one extreme parameter and referred to as
polyextreme (Ventosa, 2006). The submarine hydrothermal vents as well as the
cold layers of the deep oceans are examples of polyextreme environments,
because in addition to temperature they are also subjected to high pressure
(piezophilic environments). Not much is known about the abilities of piezophiles
to handle the high pressure, but the presence of unsaturated membrane lipids
and structural changes in the SSU ribosomal RNA genes are suggested to be
critical for life in piezophilic environments (Lauro et al., 2007).

As the following examples illuminate, extremophiles have evolved to possess
several mechanisms to keep the cell interior in balance with the surrounding
environmental stress. Another challenge is faced when the salt concentration of
the environment approaches NaCl saturation, which is a common condition in
hypersaline environments. Extreme halophiles are microorganisms able to
combat the highly osmotic conditions of their environments, which include salt
lakes, salt deposits and solar salterns (Ventosa, 2006). Also desert soil has been
found to include microorganisms such as cyanobacteria, filamentous fungi and
green algae (Lacap et al., 2011) that are able to withstand high temperature and
intense UV radiation or extremely cold and dry polar climate. Some deserts are
also hypersaline environments and sites where halophilic microorganisms can
be found (Shand and Leyva, 2008).

Since only the members of a few species survive in most extreme
environments, many extremophiles lack natural predators (Ventosa, 2006). This
increases competition between both closely and more distantly related species
thriving in environments that might not have high nutrient levels. In the absence
of predators, the ecology of extremophiles is significantly affected by their viruses, which exceed the number of their host cells at least ten-fold (Guix-Boixareu et al., 1996; Le Romancer et al., 2007; Roine and Oksanen, 2011). Viruses of extremophiles represent various incredible morphologies and most probably play a crucial role in the evolution of their hosts as well as in the cycling of carbon and nutrients in the oceans (Ortmann et al., 2006; Le Romancer et al., 2007; Rohwer and Thurber, 2009).

Figure 2. Different hypersaline environments. A.–B. Athalassohaline environment (the Dead Sea) C. Thalassohaline environment (Saltern of Eilat) D. Thalassohaline environment (Saltern of Atlit). Photo courtesy of A. Oren.

1.2 Hypersaline environments

The general definition of hypersaline environments is based on NaCl concentration that exceeds that of sea water (0.5 M/3.5 % NaCl) (DasSarma and DasSarma, 2012). However, usually hypersalinity refers to higher salt concentrations, even those close to NaCl saturation (5.5 M/35 % w/v) (Ventosa and Aralhal, 2009). Hypersaline environments (Fig. 2.) are common on our planet as they are found on all the continents. Several of them are located at
tropical or subtropical latitudes in arid or semiarid climates, but there are saline lakes even on Antarctica (Litchfield and Gillevet, 2002; Laybourn-Parry and Pearce, 2007). Hypersaline environments can generally be divided into two categories based on their genesis and ionic composition. Thalassohaline environments (Fig. 2.C-D) have originated from the oceans by the evaporation of seawater and resemble marine environments by their ion composition. The pH is slightly alkaline. Solar salterns in different parts of the world are manmade thalassohaline environments that are used for harvesting salt (NaCl) from the seawater (DasSarma and DasSarma, 2012). The Great Salt Lake of Utah (USA) provides an example of a natural thalassohaline system. Athalassohaline hypersaline environments (Fig. 2.A-B) have arisen from fresh water sources, such as mountain streams and as a result have different ion compositions (Litchfield and Gillevet, 2002). Athalassohaline salt lakes can also be highly alkaline due to high concentration of carbonates (Ventosa and Arahal, 2009). There are also hypersaline deep sea anoxic basins that contain different types of anaerobic halophilic bacteria and archaea. These waters have extremely high magnesium content, but to date little is known about them (van der Wielen et al., 2005). In addition to these aquatic environments that refer to different geographical sites in nature, hypersaline environments include salted food products and salt crystals as well as ancient salt marshes and deposits (Chaban et al., 2006).

1.2.1 Thalassohaline hypersaline environments

Solar salterns are often located at the seashore, but can also be found connected to various brine springs (Oren, 2002a, 2008). A typical saltern consists of a series of ponds with different salinities. The initial pond which is connected to the sea contains the lowest salinity. From here the water is pumped through condenser ponds as the salinity increases. The last ponds are the crystallizers (Oren, 2005a). Salterns are convenient environments to study microbiology of halophilic microorganisms, because the salinity is kept at a fairly constant value (Javor, 2002, Oren, 2005a). The Great Salt Lake is one of the best studied natural thalassohaline lake. It is the second saltiest lake in the world, after the Dead Sea (Baxter et al., 2005). The lake includes two different isolated ecosystems with drastically different salinities. The North arm of the lake is twice as saline as the South arm, due to having only a little fresh water income (Baxter et al., 2005). Thalassohaline lakes can also be found in cold environments, such as the Deep Lake in Antarctica, from which psychrophilic
Halophiles have been obtained even though the temperature of the lake surface is as low as -20 °C (DasSarma, 2006).

1.2.2 Athalassohaline hypersaline environments

The Dead Sea is probably the best studied athalassohaline hypersaline environment in the world. It is a terminal desert lake with very high concentration of divalent cations, mainly magnesium and calcium while chloride is the dominant anion. The pH is slightly acidic (at around 6) (Oren, 2002a, 2005a). The water balance of the Dead Sea is negative and the increasing salinity restricts microbial life. During rainier seasons, periodic algal blooms (corresponding for the only primary production of the lake) have been observed (Oren and Gurevich, 1995). Along with the blooms also archaea and bacteria as well as some fungi and protozoa have been reported. Also a high number of virus-like particles, especially the spindle-shaped ones, have been observed by electron microscopy (Oren et al., 1997). The increasing salinity along with decreasing water activity, high divalent cation concentration and low pH make the Dead Sea water a compromising environment even for extreme halophiles (Oren, 2005b). In order to preserve the life in the Dead Sea, there have been plans to pump water from the Red Sea to the Dead Sea following desalination by reverse osmosis (Oren, 2005b). It is not known what kind of an effect this procedure might have on the Dead Sea biology and that is why some experimental areas mixing the waters from the two sources have been established to monitor the situation, for example at Sedom (Oren et al., 2004). The experiments have revealed at least that the algal blooms may be replaced by cyanobacteria when the water of the Dead Sea is replaced 50-60 % by that of the Red Sea (Oren, 2005b).

Alkaline soda lakes are another type of athalassohaline hypersaline environments. These lakes have low amounts of divalent cations (magnesium and calcium) and instead are rich in carbonates which results in alkalinity (above pH 9) (Jones et al., 1998). Studies of different soda lakes, for example those in California, USA, and Egypt, have revealed diverse microbial communities and especially high numbers of virus-like particles (VLPs) (Jiang et al., 2004; Brum et al., 2005; Mesbah et al., 2007).

1.2.3 Halite crystals

Extensive sedimentation of rock salt or halite, as it is formally called, occurred in large basins connected to open oceans hundreds of millions of years ago due to
substantial evaporation of the sea water under warm climatic conditions (Fendrihan et al., 2006). During halite crystallization, small fluid inclusions are left inside the crystals. Often along with the fluid, some halophilic microorganisms, archaea, bacteria and algae, may be trapped inside the crystals and remain viable for long periods of time (McGenity et al., 2000). It is suggested that the trapping of halophiles inside crystals increases crystallization and the amount and size of the crystals (McGenity et al., 2000). Several halophilic archaenal strains have been isolated from salt crystals (McGenity et al., 2000; Fish et al., 2002; Fendrihan et al., 2006).

1.3 Archaea

Because archaea have the same prokaryotic cellular organization than bacteria, they were not recognized as a distinct group with a monophyletic origin, until the development of molecular biology tools during 1970’s (Woese, 2007). Already in the 1960’s it was recognized that some extremely halophilic microorganisms possessed strange ether-linked lipids (Palameta and Kates, 1966). Also photoactive pigments of halophilic archaea were studied before the discovery of archaea (Dundas and Larsen, 1962; Oesterhelt and Stoeckenius, 1971). Carl Woese and co-workers shared an interest in studying biology from a more evolutionary perspective which led into the discovery of the archaea in 1977 based on small subunit ribosomal RNA gene sequence comparison (Fig. 1.) (Woese and Fox, 1977). Methanogens were the first group of archaea to be identified followed by the extreme halophiles and thermoacidophiles (Balch et al., 1977; Fox et al., 1977; Woese et al., 1978; Balch et al., 1979). During the first years of archaenal research, it became clear that extreme halophiles and methanogens had a close evolutionary relationship whilst the thermoacidophiles were clearly different. The two major phyla, *Euryarchaeota* (comprising halophiles and methanogens) and *Crenarchaeota* (hyperthermophiles) were established in 1977 (Woese and Fox, 1977; Woese et al., 1990). At this stage, archaea were mainly considered as various extremophiles living at high temperatures and salinities or in anoxia producing methane (Woese et al., 1978). Later on, two additional phyla were suggested. *Korarchaeota* would contain phylogenetically deep branching anaerobic hyperthermophiles (Barns et al., 1996) and *Nanoarchaeota* hyperthermophilic archaenal parasites (Huber et al., 2003). To date, korarchaea are considered to be clearly different from other archaenal groups and most probably represent a distinct phylum. Nanoarchaeon
the other hand, are regarded as a euryarchaeal lineage (Brochier et al., 2005; Gribaldo and Brochier-Armanet, 2012). During recent years, it has become evident that archaea are not just extremophiles, but rather represent a significant part of the biosphere in moderate marine, terrestrial, and fresh water environments (Chaban et al., 2006; Schleper, 2007). Archaeal sequences have also been found from arthropods, termites, ruminants and even from humans (Lange et al., 2005). In 2008, a new phylum, the *Thaumarchaeota* was suggested, which would represent most of the mesophilic non-methanogenic archaea (Brochier-Armanet et al., 2008). To date, *Thaumarchaeota* represents the third confirmed archaeal phylum (Gribaldo and Brochier-Armanet, 2012).

Archaea lack a nuclear membrane and have often a rigid cell wall, which make them clearly prokaryotic. The phylogenetic analyses of rRNA gene sequences show that the eukaryotic branch diverges from that of the *Archaea* (Woese et al., 1990). Archaea have cellular features that resemble more eukaryotic counterparts, especially those related to genome replication mechanisms (Woese, 2004). The characteristic structures of archaeal RNA polymerases and transfer RNAs (tRNAs) (Polycarpo et al., 2007), as well as the lack of peptidoglycan in their cell wall more closely resembles eukaryotic features (Kletzin, 2007). The cell walls of archaea are unique and most commonly comprise a single protein or glycoprotein surface layer called S-layer (Kandler and König, 1998). The distance from the S-layer to the cytoplasmic membrane varies according to different genera, but in all cases it is closely associated with the cell membrane (Kletzin, 2007; König et al., 2007).

Archaeal membrane lipids are one of the key characteristics that distinguish archaeal cells from those of the other two domains. In general, whilst bacteria and eukaryotes have fatty acid side chains ester-linked to glycerol backbone, archaea possess isoprenoid side chains that are ether-linked to glycerol (Boucher, 2007). The most common archaeal core lipid type is sn-2,3-diphytanylglycerol diether, which is called archaeol. Many other lipid types in archaea, such as caldarchaeol, are variations of archaeol (Boucher et al., 2004). Archaeols can have one head group (monopolar) whilst caldarchaeol has two (bipolar) (Sprott, 2011). Ether lipids are generally more stable in extreme environments than ester-linked ones (van de Vossemberg et al., 1998).

### 1.3.1 *Crenarchaeota*

Crenarchaea are best known as hyperthermophiles that dwell in the hottest environments on Earth, such as volcanic hot springs, solfataras, and deep sea
hydrothermal vents (Stetter, 2002). It has even been suggested, that these heat-loving archaea belong to the most primordial species that still exist on our planet (Stetter, 2002). According to the definition, true hyperthermophiles only thrive at temperatures above 80 °C and their optimal temperatures can be as high as 113 °C (Blochl et al., 1997; Stetter, 2006). Albeit not optimally, but some crenarchaea are able to grow even at higher temperatures. The record for the highest growth temperature known to date has been measured for a crenarchaeal strain named “strain 121” according to this particular temperature, 121 °C (Kashefi and Lovley, 2003).

The crenarchaeal hyperthermophiles belong to the class Thermoprotei, and are distributed into four orders: Thermoproteales, Desulfurococcales, Sulfolobales, and Acidilobales (Oren, 2010). The order Sulfolobales contains the thermoacidophilic archaea from the genus Sulfolobus which have been well studied and developed into model organisms of extremophiles ever since Thomas Brock first isolated them in 1972 from Yellow Stone National Park (YNP) (Brock et al., 1972). Many of the aerobic chrenarchaea oxidize sulfide to sulfate, whilst the anaerobic strains reduce sulfur to sulfide (Oren, 2010). Chrenarchaea are distinguished from euryarchaea by some features discovered in genomic analyses, such as the lack of eukaryotic-like histones, DNA polymerases of the D family and certain cell division proteins that are present in euryarchaea (Matte-Tailliez et al., 2002; Ettema and Bernander, 2009). The membrane lipids of the cultured crenarchaea are mostly isoprenoid glycerol dialkyl tetraethers (De Rosa et al., 1986) that most probably function to stabilize the cell membranes at high temperatures (Boyd et al., 2011).

1.3.2 Euryarchaeota
Methanogens, extreme halophiles as well as some thermophiles are included in the phylum Euryarchaeota. The phylum consists of microorganisms with most diverse qualities dwelling in a range of different extreme and non-extreme environments (Oren, 2010). Methanogens are found in different anoxic environments such as freshwater sediments of rivers and lakes, in marine hydrothermal vents, wetlands and in the digestive track or rumen of different animals (Ferry and Kastead, 2007). Methanogenesis is a unique process known only for archaea (Bapteste et al., 2005). Methanogens are distributed into five orders, Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Methanopyrales (Ferry and Kastead, 2007). All the methanogens are obligate anaerobes that generate methane by a few different
pathways depending on the source of carbon (Bapteste et al., 2005). Methanogens are not a monophyletic group, and actually the archaea of the orders *Methanomicrobiales* and *Methanosarcinales* are more closely related to extremely halophilic archaea of *Halobacteriales* than to other methanogens (Woese and Olsen, 1986; Bapteste et al., 2005)

Extreme halophiles of *Halobacteriales* constitute a diverse group of extremely halophilic and haloalkaliphilic archaea that are usually moderately thermophilic and aerobic (Oren et al., 2009). A more detailed introduction is presented on the following pages. Aside methanogens and extreme halophiles the phylum *Euryarchaeota* includes also hyperthermophilic sulfate reducing archaea from the orders *Thermococcales* and *Archaeoglobales* (Leigh et al., 2011). *Thermococcales* includes the genera *Pyrococcus*, *Thermococcus* and *Palaeococcus* that are all obligate anaerobes isolated mostly from deep sea hydrothermal vents (Leigh et al., 2011).

![Phylogeny of archaeal domain (ribosomal proteins)](image.png)

**Figure 3.** Phylogeny of archaeal domain (ribosomal proteins). Modified from Wolf et al., 2012.
1.3.3 **Thaumarchaeota**

*Thaumarchaeota* is the newest confirmed archaeal phylum, which includes mesophiles and thermophiles (Brochier-Armanet et al., 2008; Brochier-Armanet et al., 2012). Thaumarchaea were initially considered as mesophilic or psychrophilic crenarchaea that were discovered in marine and terrestrial environments (Treuensch et al., 2005). The uncultivated archaeal strains have been detected by direct amplification of the environmental 16S ribosomal RNA (rRNA) gene sequences, as well as by in situ hybridization and the use of archaeal lipids as biomarkers (Schleper, 2007). They were suggested to have evolved from the crenarcheal hyperthermophiles. However, the SSU rRNA and genomic core analysis as well as the ribosomal protein concatenation studies by Brochier-Armanet et al., in 2008 confirmed that the mesophilic crenarchaea branch deep before the separation of crenarchaeota and euryarchaeota and thus might constitute a novel archaeal phylum, the *Thaumarchaeota* (Brochier-Armanet et al., 2008). Thaumarchaea most probably have an important role in the cycling of carbon and nitrogen in the environment, especially the ammonia oxidizing archaea in the open oceans (Pester et al., 2011). Thaumarchaea are also distinguished from euryarchaea and crenarchaea by a specific lipid called thaumarchael (Pester et al., 2011).

1.3.4 **Suggested phyla**

In addition to the three established phyla, there are two suggested ones, the *Korarchaeota* (Barns et al., 1996), and the very recent “Aigarchaeota” (Nunoura et al., 2010) (Fig. 3.).

*Korarchaeota* comprises a group of mostly uncultivated microorganisms derived from hyperthermophilic environments, originally from YNP (Barns et al., 1996). It has been thought that korarchaea are reminiscent of the ancient archaea that diverged very early in evolution (Elkins et al., 2008). Phylogenetic analyses place korarchaea as a separate deep archaeal lineage but in close affinity to the crenarchaea (Barns et al., 1996; Elkins et al., 2008). A few years ago, one korarchaeal strain, *Korarchaeum cryptofilum*, was isolated and characterized along with genome analysis (Elkins et al., 2008). This organism was confirmed to be a strict anaerobic hyperthermophile which despite the phylogenetic resemblance to the crenarchaea possessed some more euryarchaeal features related to DNA replication and cell division (Elkins et al., 2008). Korarchaeal 16S rRNA gene sequences have been found from several
hyperthermophilic, both terrestrial (hot springs) and marine (hydrothermal vents) environments (Reigstad et al., 2010).

Recently a fourth archaeal phylum, the “Aigarchaeota” was suggested based on the genome sequence of one uncultured archaeal strain designated as “Caldiarchaeum subterraneum”, which was detected in geothermal water samples. The organism was suggested to represent a new phylum due to distinct genomic features (Nunoura et al., 2010).

1.3.5 Nanoarchaea and other ultra-small archaeal cells

To date, the lineage of nanoarchaea is only represented by *Nanoarchaeum equitans*, which is a parasite of a hyperthermophilic chrenarchaeum, *Ignicoccus hospitalis* (Huber et al., 2003). *N. equitans* has the smallest known genome size (490 kb) for a cellular organism. With over 550 open reading frames (ORFs), the genome of *N. equitans* also has the highest known gene density (Huber et al., 2003; Waters et al., 2003). Both *N. equitans* and its host are strict anaerobes. Nanoarchaeal 16S rRNA genes have been amplified from the East Pacific, YNP in USA, and hydrothermal environments in Russia, indicating that these organisms could be widely distributed around the world (Hohn et al., 2002). Interestingly, recently nanoarchaeal gene sequences have also been observed in hypersaline environments in Mongolia and China, showing that nanoarchaea are represented by various phylotypes and might have a wide range of different host organisms and not just hyperthermophiles (Casanueva et al., 2008).

Lately, in addition to *N. equitans*, two other types of ultra-small archaea have been described. ARMAN cells (archaeal Richmond Mine acidophilic nanoorganisms) (~500 nm diameter) were recognized in acidic environments rich in metals by metagenomics. These cells are likely to be parasites or symbionts of other archaea (Baker et al., 2010). The other cell type referred to as nanohaloarchaea consists of small cells (~600 nm diameter) that have been revealed by metagenomics in a hypersaline lake in Australia. Apart from *N. equitans* or ARMAN cells, nanohaloarchaea seem to be free-living and represent a distinct archaeal lineage distantly related to euryarchaeal halophiles (Narasingarao et al., 2012).

1.4 Extreme halophiles

There is a huge versatility of halophilic organisms on Earth. Halophiles belong to all three domains of life and are classified in terms of optimal NaCl
concentration from slight to extreme halophiles (Oren, 2002a, 2008; DasSarma and DasSarma, 2012). In general, extreme halophiles are defined as those that thrive in NaCl concentrations from 3.4 M up to saturation (DasSarma and DasSarma, 2012). The highly saline conditions expose halophilic organisms to osmotic stress. Depending on the type of hypersaline environment, extreme halophiles can also be exposed to intense UV radiation and elevated or lowered temperatures (DasSarma, 2006). These organisms have developed several strategies how to cope with these environmental challenges.

Halophilic microorganisms have specific mechanisms to exclude sodium ions from the cytoplasm and thus keep the cell interior in osmotic balance with the external environment. Most of these are based on sodium Na+/H+ antiporters (Oren, 2008). Halophilic archaea from the order Halobacteriales and some halophilic bacteria are able to accumulate intracellular KCl to high levels in order to maintain the salt concentration of the cell interior equivalent to that of the surroundings (Oren, 1999, 2002a). This is often called the “salt-in” strategy. All the enzymes and other cellular components of microorganisms using this mechanism need to be adapted to high salinity (Oren, 1999). Many haloarchaeal proteins have acidic residues and require highly saline environment in order to function normally (Grant et al., 2001). It seems that the accumulation of potassium ions in the cell is energetically favorable and archaea might have potassium bound to their cell components and proteins. The other strategy for osmotic adaptation, which is harbored by most halophilic bacteria, the green algae, halophilic methanogens and many halotolerant microorganisms, is to synthesize small organic molecules called compatible solutes, such as glycerol, trehalose, glycine betaine, and ectoine to prevent the loss of water from the cells (DasSarma and DasSarma, 2012; Oren, 1999). Microorganisms can also take such molecules up from the environment (Oren, 1999).

1.4.1 Halophilic archaea

Extremely halophilic archaea from the order Halobacteriales are the dominant group of extremophiles in different types of hypersaline environments around the world. The order contains the family Halobacteriaceae which at the moment includes all the known extremely halophilic archaeal genera as well as some unclassified groups such as the square haloarchaea of Walsby (SHOW) (Oren, 2002a). The minimum NaCl concentration required by haloarchaea is around 1.5 M but most strains can grow even in saturated concentrations (Grant et al., 2001; Madigan et al., 2012). The amount of NaCl required for optimal growth is
around 3.5-4.5 M (Grant et al., 2001). Haloarchaeal cells are often pleomorphic, rods, or sometimes triangular or squares that grow optimally in slightly thermophilic conditions (35-50 °C). The cells usually stain Gram-negative, although strains of *Halococcus* and *Natronococcus* are Gram-variable (Grant et al., 2001). Strains of these two genera have distinct cell walls from other *Halobacteriaceae* members allowing them to survive also in low salt concentrations or in the complete absence of salts (Grant et al., 2001). The cell walls of *Halococcus* and *Natronococcus* archaee are made of a thick layer of sulfated heteropolysaccharide (Grant et al., 2001). The non coccoid cells consist of a typical S-layer where the surface glycoprotein subunits are held together by salt cations. All members of *Halobacteriaceae* are aerobic and some, such as strains from the genera *Haloferax*, *Halogeometricum*, *Halobaculum*, *Haloarcula* and *Halobacterium* may grow anaerobically in the presence of nitrate or in some cases other substances such as fumarate (Grant et al., 2001).

An interesting group within the *Halobacteriaceae* family is the square haloarchaea of Walsby (SHOW) (Burns et al., 2007). These cells with a peculiar square-shaped morphology and characteristic gas vesicles within the membrane were at first observed by A. E. Walsby in 1980 in a hypersaline pond near the Red Sea (Walsby, 1980). It took more than 20 years before the SHOW group haloarchae were successfully cultivated due to difficulties obtaining a pure culture (Bolhuis et al., 2004; Burns et al., 2004). These organisms require high amounts of NaCl (above 2.5 M) for optimal growth and many observations suggest that they are among the dominating microorganisms in several different hypersaline environments (Bolhuis et al., 2004; Burns et al., 2004, 2007).

Haloarchaea are not photosynthetic (because they do not contain chlorophyll), but some strains are able to generate energy in the form of ATP by the help of retinal pigments within the cell membranes (Oren, 2002b; Papke et al., 2003; Marshall et al., 2007; Madigan et al., 2012). These pigments include the retinal protein complex called bacteriorhodopsin (Oesterhelt and Stoeckenius, 1973) as well as the isoprenoid-derived C-50 carotenoids called bacterioruberins (Kelly, 1970). The latter help to protect the cells from intense UV radiation as well as stabilize cell membranes in highly osmotic conditions. Bacterioruberins give the cells their pinkish orange hues (Marshall et al., 2007). Bacteriorhodopsin on the other hand results in purple-red color. It is an outward proton pump that can absorb light energy and generate a proton gradient across the cell membrane with the help of ATP synthases (Marshall et al., 2007). Haloarchaea use this mode of energy conservation when supply of oxygen
becomes limited. Visually this can be seen as a change of color from pinkish orange to purple-red in the cell culture (Madigan et al., 2012). Haloarchaea have also other retinal-based pigments. One of the most commonly observed is halorhodopsin, which is a chloride pump used in the inward transport of chloride ions (Grant et al., 2001; Oren, 2009a). All these pigments contribute to the red shades of highly saline waters, but it seems that bacterioruberins are the main pigments responsible for the red color of hypersaline environments (Oren, 2009a).

Approximately 90% of the haloarchaeal lipids are polar (mostly acidic) lipids constituting the membrane lipids (phospholipids and glycolipids). The rest are of neutral lipids which are C20-C50 isoprenoids and their derivatives such as the bacterioruberin carotenoids, retinal, quinones and C30 isoprenoids (Corcelli and Lobasso, 2006; Sprott, 2011). Haloarchaeal membrane lipids are diphytanyl glycerol diethers, which usually include acidic polar headgroups such as phosphatidylglycerol (PG), phosphatidylglycerophosphate methylester (PGP-Me), phosphatidylic acid (PA) (Marshall and Brown, 1968) or sulfated sugars (Corcelli and Lobasso, 2006; Sprott, 2011). The sulfated lipids are usually not found on other archaea except haloarchaea. Archaeol is the major core lipid in halophilic archaea (Grant et al., 2001). Within the core lipids, there are (in most strains) fully saturated isoprenoid side chains that are ether-linked to the glycerol backbone (Sprott, 2011). The structure of the haloarchaeal membrane lipids results in low membrane permeability to protons and sodium ions which makes haloarchaeal membranes very stable in high NaCl concentrations as well as in elevated alkalinity (van de Vossenberg et al., 1999; Tenchov et al., 2006).

Although usually the concept “haloarchaea” refers to archaea of *Halobacteriales*, moderate and extreme halophiles as well as halotolerant archaea are also found from methanogens of the genera *Methanohalophilus* and *Methanohalobium* (Lai and Gunsalus, 1992). Apart from *Halobacteriales* strains, methanogens in hypersaline environments rely on compatible solute synthesis to cope with the high salt (Lai and Gunsalus, 1992). Methanogenic archaea as well as archaea from *Halobacteriales* have been identified by metagenomic studies from hypersaline anoxic deep sea basins with very high hydrostatic pressure. Some of the clones represent new types of deep branching euryarchaea (van der Wielen et al., 2005). Interestingly, some members of *Halobacteriales* have been described in low salinities or from completely non-saline environments. Strains belonging to the genera *Haloferax* and *Halogeometricum* that are able to grow in sea water salinity have been isolated.
from salt marsh sediments (Purdy et al., 2004). In addition, some members of the genus *Halobacterium* have been detected in non-extreme environment of boreal tree mycorrhizosphere. These archaea are anaerobes, but able to survive in aerobic conditions (Bomberg et al., 2010).

### 1.4.2 Halophilic bacteria

During the last decade, the abundance of bacteria in hypersaline environments has become evident (Antón et al., 2002). Most of the bacterial halophiles are halotolerant or moderately halophilic, but a few extremely halophilic representatives are also known. Bacterial halophiles are found within the Gram-negative phyla, *Proteobacteria*, *Bacteroidetes*, *Cytophaga*, and *Flavobacterium* (Vreeland et al., 1980; Antón et al., 2000; Vaisman and Oren, 2009) as well as the Gram-positive *Firmicutes* and *Bacillaceae* (Oren, 2002b). Anaerobic bacterial halophiles reside in the order *Halanaerobiales* (Oren, 2006). Certain halotolerant or moderately halophilic, filamentous and unicellular cyanobacteria, such as those belonging to the species *Alphanothece*, *Oscillatoria*, *Spirulina*, and *Xenococcus*, participate in the planktonic biomass of saltern ponds of intermediate salinity where they at least partly contribute to the primary production (Oren, 2012). Cyanobacterial mats are found in both thalassohaline and athalassohaline hypersaline environments and they are known to increase the evaporation of water by enhancing the absorption of solar radiation (Oren, 2012). Like most of the moderately halophilic bacteria, cyanobacteria use the accumulation of compatible solutes, such as glycine betaine, to regulate adaptation to high NaCl concentrations (Mackay, 1984).

*Salinibacter ruber* is an extremely halophilic bacterium isolated from Spanish salterns, where it likely contributes from 5-25 % of the prokaryotes (Antón et al., 2002). *S. ruber* cells contain bacterioruberin C-40 carotenoids (salinixanthin) in the cell membranes which mostly serve to protect the cells from photooxidative damage (Lutnaes et al., 2002; Oren, 2009a). This pigment also colors the cells red (Oren, 2009a). *S. ruber* uses KCl in the same way as the halophilic archaea of *Halobacteriales* to balance the cell interior with the external high salt (Antón et al., 2002). In addition, members of *Salicola* are known to be extremely halophilic and abundant in many hypersaline environments (Maturrano et al., 2006).
1.4.3 Eukaryotes in high salt
There are only a few eukaryotes known to survive in extreme salinities, but some diversity exists in lower salinities. The green algae *Dunaliella salina* is responsible for most of the primary production in hypersaline environments (Oren, 2009b). It is extremely halotolerant due to its ability to generate compatible solutes to manage with the osmotic stress. *Dunaliella* algae have beta carotenoids that protect them from UV radiation. These pigments are partly responsible for the red hues of salterns and salt lakes (Oren, 2009a). Several moderately halophilic strains of *Dunaliella* can also be found in such environments. Aside algae, also different types of halophilic yeasts dwell in hypersaline environments. These organisms are usually aerobes that use compatible solutes as a means to preserve the osmotic balance of the cell (DasSarma and DasSarma, 2012). Although extremophiles are usually associated with microorganisms, some multicellular organisms do survive in high salt. The brine shrimp *Artemia salina* feeds on the green algae. Some types of brine flies and filamentous fungi have also been observed. As a matter of fact, many hypersaline salt lakes have been established into natural conservation sites that have diverse communities of different birds such as pink flamingos (DasSarma and DasSarma, 2012).

1.5 Antimicrobial agents in hypersaline environments
The production of antimicrobial substances is known to be common for cellular organisms in all three domains (Shand and Levy, 2008). Antimicrobial substances produced by eukaryotes and bacteria have been studied already for several decades. Antibiotics were discovered in 1929 by Alexander Fleming as he observed *Penicillium* yeasts inhibiting the growth of staphylococci (Fleming, 1946). Approximately at the same time with this revolutionary discovery, strains of *Escherichia coli* were observed to produce antagonistic substances called colicins, which were the first bacteriocins to be described (O’Connor and Shand, 2002). Antimicrobial substances produced by archaea (archaeocins) were at first detected among halophilic archaea from the family *Halobacteriaceae* in 1982 (Rodríguez-Valera et al., 1982). These proteinaceous archaeocins were named halocins. About twenty years later antagonism was observed among the crenarchaeal *Sulfolobus* cells (Prangishvili et al., 2000), but to date no other types of archaeocins have been described (O’Connor and Shand, 2002; Shand and Levy, 2008).
Although halocin production is known to be a characteristic feature of different halophilic archaea (Torreblanca et al., 1994), only a few halocins have been described in detail (Table 1.). It is, however, generally assumed that most haloarchaea are halocin producers (Shand and Levy, 2008). Halocins can be divided into peptide halocins (microhalocins) with a size range of approximately 3-10 kDa and protein halocins of 30-40 kDa (O'Connor and Shand, 2002) (Table 1). The only halocins described at protein, gene and transcript level are the protein halocin H4 (Cheung et al., 1997) and the microhalocins C8 (Sun et al., 2005) and S8 (Price and Shand, 2000). The mechanism of immunity is known only for halocin C8 (Sun et al., 2005; Mei et al., 2008) and the mode of inhibition for halocin H6/H7 (Meseguer et al., 1995).

Peptide antimicrobials are known to be synthesized in two ways, either ribosomally or via multienzymatic or enzyme cascade pathways by non-ribosomal peptide synthases (Kleinkauf and von Döhren, 1990). The known halocins are ribosomally synthesized although the exact pathways of halocin synthesis are yet unknown (O'Connor and Shand, 2002). The identified halocin genes reside in megaplasmids that are common for haloarchaea. From the around 20 currently fully sequenced haloarchaeal genomes, only *Halorhabdus utahiensis* and *Haloquadratum walsbyi* do not have megaplasmids (DasSarma et al., 2009; Liu et al., 2013). In addition to halocins, these large (over 100 kbp) replicons often encode essential metabolic functions. The megaplasmid copy numbers have been observed to be the highest during the change from exponential to stationary growth and only mildly decrease afterwards (Liu et al., 2013). This is in line with the observation that most of the known halocins are produced at the highest rate when cells enter stationary phase (Torreblanca et al., 1989). Halocin sequences do not have many hits in the databases (Shand and Levy, 2008). The same applies to sulfolobicins. These extremely heat resistant archaeosins are produced by crenarchaeal *Sulfolobus islandicus*, *S. acidocaldarius* and *S. tokodaii* (Prangishvili et al., 2000; Ellen et al., 2011). Apart from halocins, the inhibitory activity of sulfolobicins is associated with membrane vesicles produced by *Sulfolobus* strains (Prangishvili et al., 2000; Ellen et al., 2011). Two genes encoding sulfolobicins have been identified in the genome of *S. acidocaldarius*. The genes are cotranscribed into two proteins, which are both required simultaneously for growth inhibition of closely related strains (Ellen et al., 2011).

As opposed to sulfolobicins, halocins are often characterized by exceptionally wide inhibitory spectra (Shand and Levy, 2008). The known halocins exhibit
either a cytostatic (growth retarding) or cytocidal (lethal) effect on the sensitive strains (Shand and Levy, 2007). Haloarchaeal protein translocation follows usually the twin-arginine translocation (Tat) pathway (Rose et al., 2002; Shand and Levy, 2007; Szabo and Pohlschroder, 2012).

Table 1. Properties of the best characterized halocins. Adapted from Shand and Levy, 2007.

<table>
<thead>
<tr>
<th>Protein halocins</th>
<th>Producer strain</th>
<th>Size (kDa)</th>
<th>Activity spectrum</th>
<th>Thermal stability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td><em>Haloferax Mediterranei</em> Xia3</td>
<td>31</td>
<td>Broad</td>
<td>&lt;50 °C</td>
<td>Rodríguez-Valera et al., 1982; Platas et al., 2002</td>
</tr>
<tr>
<td>H4</td>
<td><em>Haloferax Mediterranei</em> R4</td>
<td>34.9</td>
<td>Broad</td>
<td>&lt;60</td>
<td>Rodríguez-Valera et al., 1982; Meseguer and Rodriguez-Valera, 1985</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microhalocins</th>
<th>Producer strain</th>
<th>Size (kDa)</th>
<th>Activity spectrum</th>
<th>Thermal stability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8</td>
<td><em>Halobacterium</em> sp. A57092</td>
<td>7.4</td>
<td>Broad</td>
<td>&lt;1 h at boiling</td>
<td>Li et al., 2003</td>
</tr>
<tr>
<td>S8</td>
<td>Haloarchaeon S8a</td>
<td>3.6</td>
<td>Broad</td>
<td>&lt;24 h at boiling</td>
<td>Price and Shand, 2000</td>
</tr>
<tr>
<td>R1</td>
<td><em>Halobacterium</em> strain GN101</td>
<td>3.8</td>
<td>Broad</td>
<td>60 °C</td>
<td>Haseltine et al., 2001</td>
</tr>
<tr>
<td>H6/H7</td>
<td><em>Haloferax gibbonsii</em> Ma2.39</td>
<td>3.0</td>
<td>Broad</td>
<td>&lt;90 °C</td>
<td>Torreblanca et al., 1989; Meseguer et al., 1995</td>
</tr>
</tbody>
</table>

1.5.1 Protein halocins

Currently, only the protein halocins H1 (Platas et al., 2002) and H4 (Meseguer and Rodríguez-Valera, 1985) have been described in more detail (Table 1). Halocin H4 is a protein halocin of 34.9 kDa (Rodríguez-Valera et al., 1982; Cheung et al., 1997). *Haloferax Meditteranei*, the producer of halocin H4 has three megaplasmids, of which pHM300 (321,908 bp), contains the gene for H4 (Cheung et al., 1997). In addition to the halocin gene, this replicon contains genes for many essential metabolic functions, such as those used in the nitrate respiration. Halocin H4 is at first synthesized as a preprotein which is then cleaved into the mature active form (Cheung et al., 1997). Halocin H4 contains a signal peptide which is further removed indicating that its translocation involves the haloarchaeal sec pathway (Bolhuis, 2004). Cells sensitive to H4 eventually lyse (Meseguer and Rodriguez-Valera, 1986).
Halocin H1 is also produced by a *Hfx. mediterranei* isolate, but lacks further characterization. It is a heat-sensitive 31 kDa protein that inhibits a wide range of halophilic archaea (Platas et al., 2002; Shand and Levy, 2008). What makes H1 special among other more closely studied halocins is that the halocin production reaches its peak at the exponential growth phase of *Hfx. mediterranei* (Platas et al., 2002).

### 1.5.2 Microhalocins

Microhalocins are generally characterized as heat stable and can withstand a range of different salt concentrations as well as desalination and longtime storage at 4 °C (Shand and Levy, 2008). This type of halocins usually has a wide inhibitory spectrum and can target distantly related archaeal strains, even those from a distinct phylum (Haseltine et al., 2001). Microhalocins H6/H7, R1, C8, S8, and U1 represent the best known microhalocins (Table 1) (Shand and Levy, 2007).

Microhalocins C8 excreted by *Halobacterium* strain AS7092 (Li et al., 2003) and S8 from an uncharacterized haloarchaeon (Price and Shand, 2000), are produced as preproteins which are then cleaved into active halocins (Shand and Levy, 2008). C8 is sensitive to proteinase K treatment, but remains active after treatment with trypsin (Li et al., 2003). The same has been observed for sulfolobicins (Ellen et al., 2011). C8 has a very wide inhibitory spectrum as it inhibits also some haloalkaliphilic rods. It seems that the target of the halocin is the cell wall because it causes nicks to the cell wall of the sensitive cells which eventually lyse when in contact with the halocin (Li et al., 2003). The halocin C8 as well as its immunity protein are encoded by the same gene. The halocin is synthesized as a preprotein which contains both functions. It is suggested that the immunity protein which is anchored at the cell wall of the producer strain is able to bind multiple molecules of the halocin enhancing the immunity of the producer (Mei et al., 2008).

Halocin S8 shares a high degree of sequence similarity with another halocin called R1 (from *Halobacterium* strain GN101) (O'Connor and Shand, 2002). In contrast to C8 and many other microhalocins, S8 has a narrow host range including only *Halofex gibbonsii* and two strains of *Halobacterium* (Price and Shand, 2000). It seems that only minor changes in the amino acid sequence of halocins may lead to huge variations in stability and host range (Shand and Levy, 2008). Both halocins S8 and R1 retard the growth of the sensitive cells, but do not cause lysis (Li et al., 2003). In addition, halocin R1 also targets
Methanosarcina thermophila and some strains of Sulfolobus (Haseltine et al., 2001).

Microalocin H6/H7 secreted by Haloferax gibbonsii strains is very stable at different salinities and temperatures (Torreblanca et al., 1989). Halocin H6/H7 targets the Na+/H+ antiporter of the sensitive Halobacterium salinarum strain (Meseguer et al., 1995). The inhibition of this antiporter results in lysis of the cells.

### 1.6 Viruses of Extremophiles

Viruses are tiny parasites consisting of nucleic acid (DNA or RNA), protein and sometimes lipids. For most viruses, the viral genome is surrounded by a proteinaceous structure called capsid, which protects the genome and enables specific attachment to the host cell. Capsid structures are highly conserved and the essential characteristic which distinguishes viruses from other self-replicating genetic elements such as plasmids (Krupovic and Bamford, 2010). Some viruses have an internal lipid membrane or an external envelope that are often associated with entry and egress mechanisms (King et al., 2012). Lipids can also be found as modifications in some viral proteins (Hruby and Franke, 1993; Pietilä et al., 2012). Virus morphotypes are diverse and in many cases unique in nature. All viruses known to date totally depend on their host cells for reproduction.

While medically important viruses are considered as the hot topic due to their ability to cause diseases, lately environmental virology has gained more attention due to the huge number of viruses in the biosphere and their impact on biological and geochemical cycles (Suttle, 2007). It has been estimated that the biosphere contains approximately $10^{31}$ virus particles (Suttle, 2007), and viruses outnumber cells at least by an order of magnitude. Consequently, viruses have a great impact on cellular evolution, as stated in Bamford, 2003: “Cellular life is bathing in a virtual sea of viruses, possibly creating the highest selective pressure they encounter.” (Bamford, 2003). Viruses are also considered as important contributors to the cycling of nutrients, regulation of host abundance, and might even have contribution to climate change (Rohwer and Thurber, 2009; Danovaro et al., 2011). A large portion of the virosphere can be found in the oceans, which cover as much as 70% of the Earth’s surface (Suttle, 2005). Such high numbers as $10^7$-$10^{10}$ virus-like particles (VLPs) in a liter of sea water have been estimated and in marine sediments the abundance is even 100 to 1000-fold.
higher (Danovaro et al., 2011). This implies that practically all sea organisms are infected by marine viruses, which play an important part in the ecosystem as they influence the mortality of sea organisms both in surface waters and in great depths (Suttle, 2005). Virus-host dynamics are also important in the control of freshwater ecosystems (Colombet and Sime-Ngando, 2012; Hewson et al., 2012).

Although viruses of extremophiles have been studied only a fairly short period of time, they are known to exist in all different types of extreme environments (Le Romancer et al., 2007; Orange et al., 2011). Aquatic extreme environments including hot springs, hydrothermal vents, sea ice and hypersaline lakes are dominated by prokaryotic viruses and the most extreme niches by archaean viruses (Le Romancer et al., 2007). Hyperthermophilic hot springs for example are known to contain approximately $10^6$ archaean and bacterial viruses in a milliliter that are suggested to influence the community structure of hyperthermophiles (Breitbart et al., 2004). Submarine hydrothermal vent environments can contain $10^5$-$10^8$ ml$^{-1}$ polyextremophilic viruses affecting microbial mortality (Ortmann and Suttle, 2005). Psychrophilic environments harbor extremely high virus counts as well, as $10^6$-$10^8$ VLPs in a milliliter have been detected in Arctic sea ice (Maranger et al., 1994) and high numbers of different virus types can be found from both fresh water and marine environments of Antarctica (Guixa-Boixareu et al., 2002; Borriss et al., 2003; Pearce and Wilson, 2003; Lopez-Bueno et al., 2009). Compared to the other extreme aquatic environments, hypersaline waters contain the highest numbers of viruses, as approximately $10^9$ VLPs in a milliliter have been described in both thalassohaline (Guixa-Boixareu et al., 1996) and athalassohaline environments (Jiang et al., 2004).

Virus abundance in soil seems to be even higher than in aquatic environments (Srinivasiah et al., 2008) and the viruses in different soil types seem to be taxonomically diverse both locally and geographically (Fierer et al., 2007). However, very few reports exist about viruses in extreme soil environments. Extremophilic head-tailed bacteriophages that are very resistant to UV radiation, desiccation, and temperature variations between thermal and psychrophilic range have been isolated from Sahara desert soil (Prigent et al., 2005), but there are scarce reports about viruses from hypersaline soils and none from salt crystals. Based on artificial fossilization of extremophilic viruses in silica, it has been suggested that viruses could be fossilized in ancient rocks in the same way as microorganisms (Orange et al., 2011).
The extreme niches of the planet are in many cases fairly isolated environments that are suggested to resemble the conditions of the early Earth. Extremophiles are thus considered to be similar to the early cellular life forms and their viruses could be even more ancient (Forterre, 2006). It has been suggested that viruses were already infecting the cellular organisms that existed before the separation of the three domains of life (Bamford, 2003). Because viruses are in constant interaction with their host cells, many viral functions related to cell entry and replication are subjected to rapid evolution. Virion structural and assembly principles on the other hand, constitute the highly conserved “viral self” element that can be used to group viruses into different structure-based viral lineages indicating common ancestry (Bamford et al., 2002; Bamford, 2003). Protein crystallography, electron cryo-microscopy (cryo-EM) and bioinformatic analyses have revealed that many viruses, regardless of the host organism and replication mechanism, share the same major capsid protein (MCP) fold and virion architecture (Benson et al., 1999, 2004; Abrescia et al., 2010; Krupovic and Bamford, 2010; Abrescia et al., 2012). To date, four different viral lineages based on four identified MCP folds, have been established and few more are thought to exist (Abrescia et al., 2012) indicating that viruses have a polyphyletic origin (Bamford, 2003).

1.7 Archaeal viruses

Before this study (see below results and discussion), only about 50 archaeal viruses have been described corresponding approximately 1% of the known viruses (Ackermann, 2007). The known archaeal virus morphotypes (prior to this study) are presented in Fig 4. The first archaeal viruses resembled head-tailed bacteriophages of the viral families *Myoviridae* and *Siphoviridae* and were isolated from the spontaneous lysates of the haloarchaeal host *Halobacterium salinarum* a few decades ago (Reiter et al., 1988; Torsvik and Dundas, 1974). To date, in addition to extreme halophiles, the isolated archaeal viruses infect creanarchaeal hyperthermophiles as well as methanogens and thermophiles belonging to *Euryarchaeota* (Pina et al., 2011; Roine and Oksanen, 2011). No viruses have yet been identified for the proposed thaumarchaea (Brochier-Armanet et al., 2008). All the described archaeal viruses have dsDNA genomes, except for *Halorubrum* pleomorphic virus 1 (HRPV-1) and *Aeropyrum* coil-shaped virus (ACV), which possess ssDNA genomes (Pietilä et al., 2009; Mochizuki et al., 2012). To date, no RNA viruses have been found infecting
archaea, although metagenomic analyses from YNP hot springs indicate that they might exist (Bolduc et al., 2012).

Figure 4. Different morphotypes of archaeal viruses. Letters on the right indicate the host domain of specific virus morphotypes based on the viral lineage hypothesis: A: Archaea, B: Bacteria, E: Eucarya.

As opposed to euryarchaeal viruses, no head-tailed viruses are known to infect crenarchaea. On the contrary, viruses of crenarchaea are famous for their unique morphotypes including spindle-shaped, filamentous, bottle-shaped, and
two-tailed particles (Fig. 4.) (Pina et al., 2011). These viruses have been isolated from terrestrial hot springs of YNP and similar environments in other parts of the world, such as Iceland, Russia and Japan (Rice et al., 2001; Ortmann et al., 2006). The known crenarchaeal viruses infect hyperthermophiles from the genera *Acidianus*, *Sulfolobus*, *Stygiolobus*, *Thermoproteus*, *Pyrobaculum*, and *Aeropyrum* (Pina et al., 2011). The genome sequences of crenarchaeal viruses are not related to other viruses (Prangishvili and Garrett, 2004; Ortmann et al., 2006).

Although the first viruses infecting halophilic archaea (haloviruses) were obtained from host cultures, salted food products and flagellar preparations (Reiter et al., 1988), lately the isolated haloviruses mostly come from different hypersaline environments including solar salters and salt lakes (Roine and Oksanen, 2011). The head-tailed myovirus and siphovirus morphotypes dominate in halovirus isolations, although direct EM of hypersaline water samples highlights the abundance of spindle-shaped VPLs (Guixa-Boixareu et al., 1996; Sime-Ngando et al., 2010). Halovirus morphotypes resembling those described for crenarchaeal viruses such as spindle-shaped and filamentous are commonly observed by transmission electron microscopy (TEM) analyses and also unusual morphotypes, such as star-shaped, bacilliform and “tadpole”-shaped particles seem to be present in salt lakes and salterns (Oren et al., 1997; Sime-Ngando et al., 2010). The dominance of the head-tailed morphology among the isolated haloviruses is suggested to result from biased culturing methods that would favor the isolation of lytic viruses (Ackermann and Prangishvili, 2012). The hosts of the described haloviruses belong to the genera *Halobacterium*, *Haloarcula*, *Halorubrum*, *Haloferax*, *Natralba*, and *Natrinema* (Pina et al., 2011).

In addition, viruses or VLPs have been described infecting the anaerobic thermophilic deep sea euryarchaea belonging to the order *Thermococcales* (Geslin et al., 2003) and a few viruses are known to infect methanogens (Meile et al., 1989; Wood et al., 1989; Pfister et al., 1998).

1.7.1 Icosahedral viruses

Icosahedral virion morphology is universal for viruses infecting organisms from all three domains of life (Fig. 4.). Nevertheless, prior to this study, only four archaeal icosahedral viruses are known at the moment (see Results and Discussion). From these *Sulfolobus* turreted icosahedral virus (STIV) and *Sulfolobus* turreted icosahedral virus 2 (STIV2) infect crenarchaea (Rice et al.,
2004; Happonen et al., 2010), while SH1 and SNJ1 are viruses of euryarchaeal extreme halophiles (Porter et al., 2005; Zhang et al., 2012).

1.7.1.1 Icosahedral viruses of crenarchaea

STIV, which was the first archaeal icosahedral virus to be characterized, was isolated from a thermal acidic spring in YNP (Rice et al., 2004). A special feature of the virus is the occurrence of 24 nm long turret-like appendages at the vertices of the virion (Fig. 4.). These structures are suggested to play a role in host cell recognition and attachment. The turrets include narrow channels that connect the virion interior with the exterior and are possibly involved in viral nucleic acid translocation (Rice et al., 2004). STIV genome consists of a 17.6 kbp circular dsDNA molecule and encodes 36 predicted open reading frames (ORFs) (Rice et al., 2004; Maaty et al., 2006). Apart from most described crenarchaeal viruses, STIV is a lytic virus (Ortmann et al., 2008). Transmission electron microscopy of infected Sulfolobus cells revealed that during STIV assembly, immature particles containing the lipid membrane and the MCP are produced followed by the insertion of turrets. These intermediates are later packaged with the viral genome prior to lysis (Brumfield et al., 2009). Same type of an assembly process has been observed for e.g. bacteriophage PRD1 (Mindich et al., 1982). The release of STIV from the host S. solfataricus involves the formation of seven-sided pyramidal protrusions, which lead into the lysis of the host through disruption of the S-layer (Brumfield et al., 2009).

STIV2 and its host Sulfolobus sp. G4ST-2 were isolated from a geothermal area in Iceland (Happonen et al., 2010). STIV and STIV2 share similarities in genome organization and virion structure and are able to infect the same host. The main differences between the two viruses lie in the structure of the turrets (Happonen et al., 2010).

1.7.1.2 Icosahedral viruses of euryarchaea

The first euryarchaeal icosahedral virus, SH1, infecting Haloarcula hispanica was isolated from a saline lake (Serpentine Lake) in Australia (Fig. 4.) (Porter et al., 2005). SH1 is a lytic virus with a linear dsDNA genome of 30.9 kbp and 53 ORFs (Bamford et al., 2005a; Porter et al., 2005). The virus contains an internal lipid membrane similar to STIV and PRD1 (Kivelä et al., 2006) and as usual for internal membrane-containing viruses, SH1 acquires its lipids selectively from its host during assembly (Bamford et al., 2005a). Of the four identified major lipids of Har. hispanica, PG, PGP-Me, PGS, and triglycosyl glycerediether
(TGD), SH1 incorporates only the first three and in different proportions than in the host (Bamford et al., 2005a). SH1 requires high concentration of NaCl for stability (Kivelä et al., 2006). The spike complexes at the virion vertices (Fig. 4.) are suggested to be associated with host receptor recognition (Jäälinoja et al., 2008).

Recently, another icosahedral halovirus, SNJ1, was found from a lysogenic strain, Natrinema sp. J7-1 by mitomycin C induction (Zhang et al., 2012). SNJ1 forms plaques on another strain, Natrinema sp. J7-2. The genomic sequences of the two strains are identical, but the strain J7-1 harbors a plasmid pHH205, which was confirmed to be SNJ1 provirus. Previously, SNJ1 was described as a member of Siphoviridae (Mei et al., 2007), but the recent study proved that SNJ1 is a temperate icosahedral virus which resides as a circular plasmid in the cytoplasm of Natrinema sp. J7-1 (Zhang et al., 2012).

1.7.1.3 Lineage of PRD1-like viruses

The structure-based lineage of PRD1-like viruses contains icosahedral viruses, which share the same double beta barrel MCP fold and virion architecture. These viruses (except for adenovirus) also contain an internal lipid membrane. Bacterial and eukaryotic viruses PRD1, Bam35, adenovirus, Paramecium bursaria Chlorella virus 1 (PBCV-1), PM2, Mimivirus and its virophage (Sputnik), as well as vaccinia virus have been grouped into this lineage (Xiao et al., 2009; Sun et al., 2010; Bahar et al., 2011; Abrescia et al., 2012). The crystal structure of STIV MCP revealed the same double beta barrel fold making it the first archaean virus belonging to this lineage (Khayat et al., 2005). In addition to the MCP fold and virion architecture, these viruses have similar DNA packaging ATPases (Abrescia et al., 2012).

According to cryo-EM microscopic analyses, the archaean viruses SH1 and STIV2 might also belong to the PRD1-like virus lineage (Jäälinoja et al., 2008; Happonen et al., 2010). Putative packaging ATPase sequence comparison with archaean, eukaryotic, and bacterial viruses, as well as the presence of host-derived lipids, strongly suggest that SNJ1 is also a member (Zhang et al., 2012). However, these viruses (apart from STIV2, which seems to be similar to STIV) constitute a distinct subgroup within the lineage characterized by the presence of two MCPs instead of one. This subgroup also includes bacteriophages infecting Thermus strains, two haloviruses (see Results and Discussion), and two euryarchaeal proviruses (Krupovic and Bamford, 2008; Jalasvuori et al., 2009; Abrescia et al., 2012).
1.7.2 Head-tailed viruses

Head-tailed viruses represent the most common morphology known to date for prokaryotic viruses (Ackermann and Prangishvili, 2012). According to current classification (King et al., 2012), there are three types of head-tailed viruses grouped into the families *Myoviridae*, *Siphoviridae*, and *Podoviridae* of the viral order *Caudovirales* (Maniloff and Ackermann, 1998; King et al., 2012). All the members of this order have linear dsDNA genomes and do not contain lipids. Myoviruses (exemplified by bacteriophage T4) represent the most complex head-tailed morphotype, which consists of an icosahedral or prolate head, a neck structure, and a contractile tail which contains a sheath and a central tube (Fig. 4.). Siphoviruses have non-contractile tails that are longer than those of the other two types (Fig. 4.). Podovirus tails are very short and non-contractile. In the case of bacteriophages, siphoviruses are the most common (61 %), followed by myoviruses (25 %) and podoviruses (14 %) (Ackermann, 2006). Archaea, however, seem to have myoviruses as the dominant head-tailed morphotype (Ackermann and Prangishvili, 2012). Prior to this study, no podoviruses have been characterized for archaea (see Results and Discussion).

It has been speculated that head-tailed viruses might be absent in the most hyperthermophilic environments. Some observations, based on direct EM micrographs of enriched hyperthermophilic water samples, however, show icosahedral tailed VLPs reminiscent of sipho- and podoviruses (Rachel et al., 2002). Recently also a provirus with a gene encoding an MCP similar to head-tailed bacteriophages and eukaryotic herpesviruses, was found in the *Sulfolobus solfataricus* genome (Heinemann et al., 2011).

1.7.2.1 Archaeal myoviruses

The best characterized member of archaeal myoviruses is ΦH, which was originally isolated from a spontaneous lysate of the host (*Hbt. salinarum*). It is a temperate virus with a genome size of 59 kbp (Schnabel and Zillig, 1984). The genome of ΦH contains a specific L-region which can circulate into a plasmid form. The virus does not integrate into the host genome, but replicates as a plasmid in the cytoplasm. The plasmid containing the L-region gives the host immunity to ΦH (Stolt and Zillig, 1992, 1994). The L-region of ΦH shares a high degree of sequence similarity with a central genomic region of another temperate myovirus, ΦCh1 infecting *Natricalba magadii*, which is a haloalkaliphilic member of the *Halobacteriaceae* (Klein et al., 2002). The two viruses also have similar structural proteins and they both use head-full DNA packaging.
mechanism. The main difference lies in the mode of the temperate life style. While ΨH replicates as a plasmid, ΦCh1 integrates into the host genome (Witte et al., 1997). ΦCh1 is the only archaeal virus which contains host-derived RNA in its virion (Witte et al., 1997).

Other well-studied archaeal myoviruses include the virulent HF1 and HF2, isolated from the same saltern pond in Australia (Nuttall and Dyall-Smith, 1993). These viruses contain fairly similar protein profiles and related linear dsDNA genomes, but have very different host ranges (Nuttall and Dyall-Smith, 1993). HF1 has a broad host range infecting different members of Halobacteriaceae, while HF2 only infects Halorubrum coriense and Hr. saccharovorum (Tang et al., 2002, 2004). Both viruses are lytic. The genome of HF2 has been fully sequenced, which revealed that the virus shares homologous DNA fragments with Mycobacterium and Enterobacteria phages, as well as with bacteria from the genera Mycobacterium, Listeria, Thermoplasma, and Shinorhizobium (Tang et al., 2002). The virus is not related to temperate haloarchaeal myoviruses mentioned above or any other known viruses except for HF1 (Tang et al., 2002). HF1 and HF2 are the only known archaeal head-tailed viruses that encode their own DNA polymerases (Nuttall and Dyall-Smith, 1993).

1.7.2.2 Archaeal siphoviruses

Most of the characterized archaeal siphoviruses infect methanogens. Probably the best studied ones are ΨM1 of Methanobacterium marburgensis (formerly Methanobacterium thermoautotrophicum) and its deletion mutant, ΨM2 (Jordan et al., 1989). ΨM1 was shown to use the headful packaging method for its circularly permuted 30.4 kbp linear dsDNA genome (Jordan et al., 1989). The virus is also able to pack plasmid DNA from the host into virus particles (Meile et al., 1989). ΨM1 is able to transduce genetic markers making it the only known transducing archaeal virus (Meile et al., 1990). Another well characterized siphovirus, ΨM100, was found as a prophage in the genome of Methanobacterium wolfeii and is highly similar to ΨM2 (Luo et al., 2001). In addition, a few other head-tailed viruses have been described infecting methanogens, but they are not extensively studied (Stedman et al., 2006).

The described haloarchaeal siphoviruses include Hh1 and Hh3 isolated from salted fish sauce, Ja1 and S45 obtained from Jamaican salterns, ΦN from Halobacterium salinarum, B10 infecting a Halobacterium strain and BJ1 from a salt lake in Mongolia infecting Halorubrum saccharovorum, but these isolates
lack further characterization and some of them are probably lost (Dyall-Smith et al., 2003).

1.7.2.3 Lineage of HK97-like viruses

The lineage of HK97-like viruses includes head-tailed bacteriophages belonging to the order Caudovirales, as well as eukaryotic herpes viruses (Abrescia et al., 2012). This fold was first observed for the lambda-like bacteriophage HK97 (Wikoff et al., 2000). Afterwards, this MCP type has been detected also for P22, Φ29, and T4 phages. Interestingly, the MCP of Herpes simplex virus also contained the HK97 fold (Baker et al., 2005; Bamford et al., 2005b).

A few years ago, nine archaeal proviruses with putative HK97 MCP fold were identified in the genomes of Hrr. lacusprofundi, Natrialba magadii and methanogens belonging to the order Methanococcales and Methanosarcinales. The same study indicated that the MCP of the ΦCh1 has the HK97-fold (Krupovic et al., 2010). Sequence comparison studies revealed that archaeal head-tailed viruses including these proviruses and bacteriophages of Caudovirales use similar strategies for capsid and tail assembly and genome packaging (Krupovic et al., 2010). It is thus considered that archaeal head-tailed viruses belong to this lineage.

1.7.3 Pleomorphic viruses

Recently, a novel type of a haloarchaeal virus infecting a Halorubrum strain was isolated from a solar saltern. This virus, Halorubrum pleomorphic virus 1 (HRPV-1), has a roughly spherical morphology with spikes on the virion surface (Fig. 4.). It is the first described archaeal virus with an ssDNA genome (circular 7048 nt) (Pietilä et al., 2009). In addition, another virus, Haloarcula hispanica pleomorphic virus 1 (HHPV-1), with a similar morphology, but a circular dsDNA (8082 bp) genome, has been described from a similar hypersaline environment (Roine et al., 2010).

The pleomorphic virus architecture portrayed by HRPV-1 and HHPV-1 is very simple consisting of a membrane vesicle, which encloses the genome without associated nucleoproteins (Fig. 4.) (Pietilä et al., 2010). The virion of HRPV-1 and HHPV-1 consists of two major structural proteins. The larger protein represents the spike and the smaller is an internal membrane protein (Pietilä et al., 2009; Roine et al., 2010). The composition of the lipids of HRPV-1 and HHPV-1 resembles that of their host indicating that they are acquired non-selectively.
On the contrary to many described haloarchaeal viruses, HRPV-1 and HHPV-1 do not exit their host cells by lysis as viruses are produced continually until the host growth ceases (Pietilä et al., 2009; Roine et al., 2010). The presence of a lipid membrane and the non-lytic life cycle of the viruses suggest that they exit their host cells by budding mechanism. The entry is suggested to occur by fusion mediated by the viral spikes (Pietilä et al., 2009, 2010; Roine et al., 2010). However, further studies are required to assess these questions.

HRPV-1 and HHPV-1 share similarities in their genome organization as well as amino acid sequences despite the different genome types (Roine et al., 2010). These virus genomes have also been found to be related to a pHK2 plasmid from *Haloferax* sp. and genomic regions of *Haloferax volcanii* and *Halomicrobium mukohataei* that are suggested to be proviruses (Roine et al., 2010; Roine and Oksanen, 2011). Recently, also a plasmid from *Haloquadratum walsbyi* was found to be related to HRPV-1 (Dyall-Smith et al., 2011). In addition, sequence similarities have been detected between the genome of His2 virus and in two haloarchaeal genomes (*Haloarcula marismortui* and *Natronomonas pharaonis*) (Pietilä et al., 2010).

### 1.7.4 Linear viruses

Linear viruses have been observed to be one of the most abundant virus types in hyperthermophilic environments (Prangishvili and Garrett, 2004). This type of VLPs has been observed by direct EM also in hypersaline environments, but no viruses have yet been identified (Sime-Ngando et al., 2010). All archaeal linear viruses known to date infect crenarchaee and have been classified into two families, *Rudiviridae* and *Lipothrixviridae* (Fig. 4.) (Prangishvili et al., 2006a; Pina et al., 2011). Rudiviruses are rod-shaped and non-enveloped, while lipothrixviruses are filamentous and enveloped. All virus isolates from both families contain linear dsDNA genomes (Pina et al., 2011). To date, linear viruses with this genome type have only been observed for archaea (King et al., 2012).

#### 1.7.4.1 The non-enveloped rod-shaped viruses of *Rudiviridae*

The four described rudiviruses include: *Sulfolobus islandicus* rod-shaped virus 1 (SIRV1) (Zillig et al., 1994) and 2 (SIRV2) (Zillig et al., 1998; Prangishvili et al., 1999), *Acidianus* rod-shaped virus 1 (ARV1) (Vestergaard et al., 2005), and *Stygiolobus* rod-shaped virus (SRV) (Vestergaard et al., 2008a). The rudiviruses consist of an approximately 600-900 nm long superhelix formed by viral DNA and a DNA binding protein. In addition, there are three tail fibers at both ends of
the rod structure (Prangishvili et al., 1999). Genomes of SIRV1 and SIRV2 are linear dsDNA molecules of 32.3 and 35.8 kbp in length, respectively. The genomes have similar organization and a low G+C content (Kessler et al., 2004). Rudiviruses are able to develop a carrier state with their hosts and are extremely stable in hyperthermophilic conditions (Pina et al., 2011).

Rudivirus SIRV2 life cycle has been studied in more detail and surprisingly the virus was found to be lytic on the contrary of what was previously proposed (Prangishvili et al., 1999; Bize et al., 2009). SIRV2 particles were observed to assemble in the host cytoplasm and induce development of pyramidal inclusions protruding from the cell envelope as has been observed around the same time also for the icosahedral STIV (Bize et al., 2009; Brumfield et al., 2009). The viral protein (P98) is responsible for the formation of the pyramids (Quax et al., 2010).

1.7.4.2 The enveloped filamentous viruses of Lipothrixviridae

Lipothrixviruses are flexible enveloped filaments of approximately 900-2000 nm in length and are tapered from the ends. The width of the lipothrixvirion is 24 nm, the same as for rudiviruses. The nine known representatives are: Thermoproteus tenax virus 1 (TTV1), Sulfolobus islandicus filamentous virus 1 (SIFV), and Acidianus filamentous viruses (AFV) 1, 2, 3, 6, 7, 8, and 9, (Janekovic et al., 1983; Bettstetter et al., 2003; Bize et al., 2008; Häring et al., 2005a; Vestergaard et al., 2008b). The genome sizes of the lipothrixviruses are around 15-40 kbp (Vestergaard et al., 2008b). They differ from each other by their specific terminal structures at virion ends, which can consist of a group of thin fibers, claw-like structures or bottle brush-like structures. In addition, these viruses have unrelated genome sequences. Due to these differences, the family Lipothrixviridae is divided into four genera called Alpha-, Beta-, Gamma-, and Deltalipothrixvirus (Pina et al., 2011). Most of the described lipothrixviruses belong to the Betalipothrixvirus genus. Betalipothrixvirus AFV3 virions were found to contain a 3 nm thick cylinder-like envelope and an inner core consisting of two zipper-like ordered protein subunit rows (Vestergaard et al., 2008b). It has been suggested, that rudiviruses and lipothrixviruses could be assigned to a common viral order “Ligamenvirales” based on comparative genomic analysis and structural similarities of viral MCPs indicating possibly common ancestry (Prangishvili and Krupovic, 2012).
1.7.5 Spindle-shaped viruses

Spindle-shaped viruses are suggested to be unique for archaea. Spindle-shaped VLPs are commonly observed in hypersaline aquatic environments by TEM (Guixa-Boixareu et al., 1996; Oren et al., 1997; Sime-Ngando et al., 2010) and this virus type seems to be also highly abundant in hyperthermophilic environments (Rice et al., 2001). The described spindle-shaped viruses are classified into two families, Fuselloviridae and Bicaudaviridae (infecting crenarchaea), and to one genus, Salterprovirus (Fig. 4.) (infecting euryarchaeal extreme halophiles) (Pina et al., 2011). In addition, some unclassified spindle-shaped viruses or VLPs have been described for euryarchaeal thermophiles and methanogens (Wood et al., 1989; Geslin et al., 2003). It has been proposed, that the different types of spindle-shaped viruses introduced here would constitute a novel structure-based viral lineage (Pietilä et al., 2012).

1.7.5.1 Spindle-shaped viruses of crenarchaea

Currently, the family Fuselloviridae contains only one genus, Fusellovirus, with Sulfolobus spindle-shaped virus 1 (SSV1) as the type species (King et al., 2012). However, at least nine other fuselloviruses infecting crenarchaea of the genera Sulfolobus and Acidianus have been described (Pina et al., 2011; King et al., 2012). The virions are approximately 50-60 nm wide and 100 nm long spindles with a short tail at the tapered end (Fig. 4.) (King et al., 2012). The viruses Acidianus spindle-shaped virus 1 (ASV1) and Sulfolobus spindle-shaped virus 6 (SSV6) are more pleomorphic in shape (Redder et al., 2009). In addition, elongated forms of SSV1 are often observed in TEM preparations of the virus. The genomes of all known fuselloviruses are circular dsDNA molecules (King et al., 2012).

The family Bicaudaviridae only comprises one virus, Acidianus two tailed virus (ATV) (Fig. 4.) (Pina et al., 2011). This virus is able to develop two long tails at both pointed ends of the spindle-shaped virion when in extracellular state (the viruses are released from the cell without visible tails) (Prangishvili et al., 2006b). This type of behavior has not been observed for any other virus and ATV seems to be an exceptional virus in many ways. The virus contains four putative transposable elements in its circular (62.7 kbp) dsDNA genome and encodes over 70 predicted proteins (Prangishvili et al., 2006b). One of these proteins is likely a viral integrase which allows ATV to integrate into the host chromosome during lysogenic state. One unclassified virus, Sulfolobus tengchongensis
spindle-shaped virus 1 (STSV1) with a tail of variable length might be somewhat similar to ATV (Xiang et al., 2005), but remains unclassified.

Recently, *Aeropyrum pernix* spindle-shaped virus 1 (APSV1) was discovered at first as a provirus in the genome of *A. pernix* from which it was induced by lowered oxygen supply (Mochizuki et al., 2011). APSV1 virions are 200 nm long spindles with variable length tails. Most virions contain one tail, but two-tailed forms similar to ATV have been reported. One tail containing particles have three filaments protruding from one end. The genome of APSV1 is a 38.0 kbp circular dsDNA molecule. The virus encodes an integrase of the SSV-type and is morphologically most similar to STSV1 (Mochizuki et al., 2011).

### 1.7.5.2 Spindle-shaped viruses and VLPs of euryarchaea

For haloarchaea, only one spindle-shaped virus, His1, has been described so far. His1 is the type species and the only current member of the genus *Salterprovirus* (Pina et al., 2011). Opposite to fuselloviruses, the genome of His1 is a linear dsDNA molecule of 14.9 kb (Bath and Dyall-Smith, 1998). This virus was isolated from the saltern in Victoria, Australia (Bath and Dyall-Smith, 1998). Initially His2, which was characterized simultaneously with His1, but isolated from a different environment, was considered to be spindle-shaped as well (Bath et al., 2006). However, the virus was later shown to represent a different morphology (see Results and Discussion). His1 virions are clear spindles in the negative staining EM similar to fuselloviruses (Pina et al., 2011). The length of the virion is approximately 75 nm while the width is less than 50 nm. In addition, there is a short tail at the tapering end of the virion (Fig. 4.). The virus infects *Har. hispanica* and is non-lytic only slightly retarding host growth at the end of the growth phase (Pietilä et al., 2012). Interestingly, His1 was found to tolerate a wide range of salinities and was infective even at low salinity (100 mM) His1 virion has two major structural proteins (about 8 and 11 kDa) that are encoded by the same gene. The 11 kDa protein band was confirmed to be lipid-modified version of the 8 kDa protein. However, the virus does not have a lipid membrane (Pietilä et al., 2012).

Spindle-shaped VLPs of the deep-sea euryarchaeal anaerobes from the genera *Pyrococcus* and *Thermococcus* (*Thermococcales*) are most probably viruses. *Pyrococcus abyssi* virus 1 (PAV1) resembles morphologically fuselloviruses as does *Thermococcus prieurii* virus 1 (TPV1) (Geslin et al., 2003; Gorlas et al., 2012). Similar VPLs have also been characterized for methanogens.
A3 is a spindle-shaped VLP of *Methanococcus voltae* (Wood et al., 1989; Meile et al., 1989).

### 1.7.6 Unique morphotypes of crenarchaeal viruses

Crenarchaeal viruses are famous for their unique morphologies that have not been encountered among other viruses. Most of these viruses have been isolated from the acidic and neutral geothermal hot springs of YNP and similar environments (Ortmann et al., 2006).

#### 1.7.6.1 Spherical viruses

The family *Globuloviridae* contains the enveloped spherical viruses *Pyrobaculum* spherical virus (PSV1) (Häring et al., 2004) and *Thermoproteus tenax* spherical virus (TTSV1) (Fig. 4.) (Ahn et al., 2006). PSV1 and TTSV1 have linear dsDNA genomes of 28.3 and 21.6 kbp respectively, which are tightly packed inside a superhelical nucleoprotein core. The virions of TTSV1 and PSV1 are approximately 70 and 100 nm by diameter respectively, and are surrounded by a lipid envelope derived from host cell. Globuloviruses seem to have non-lytic life cycles and are able to establish a carrier state within the host cells, but do not integrate into the host chromosome. The hosts of these viruses are strict anaerobes and no plaque assay exists for the viruses (Häring et al., 2004; Ahn et al., 2006). PSV was originally isolated from YNP and TTSV1 from hot springs of Siteri geothermal area in Indonesia. To date these two isolates represent the only known enveloped DNA viruses with a helical nucleoprotein core (Häring et al., 2004; Ahn et al., 2006).

#### 1.7.6.2 Droplet-shaped viruses

*Sulfolobus neozealandicus* droplet-shaped virus (SNDV) is the only current member of the family *Guttaviridae* (Arnold et al., 2000). SNDV was isolated from a *Sulfolobus* strain derived from Indonesian solfataric fields. The virus exists in a stable carrier state with the host. The 110-185 nm long virions consist of a spiral-like ordered core covered by helical or stacked coat with tail fibers at the pointed ends (Fig. 4.). The structure of the coat and the presence of tail fibers at the tapered end are the main morphological features that distinguish SNDV from the spindle-shaped fuselloviruses (Fig. 4.). SNDV genome is 20 kbp circular dsDNA. The virus is non-lytic and has not been observed to integrate into host chromosome (Arnold et al., 2000).
A couple of years ago a novel virus that morphologically resembles SNDV was described infecting *Aeropyrum pernix*, a hyperthermophile with an optimal growth temperature at 90-95 °C. This virus called *Aeropyrum pernix* ovoid-shaped virus 1 (APOV1) has a 70 nm long ovoid or droplet-shaped morphology characteristic to the *Guttaviridae* family (Mochizuki et al., 2011). APOV1 was isolated by induction from *A. pernix* by lowered oxygen supply. The host contains a proviral genomic region which corresponds to the circular 13.8 kbp dsDNA genome of APOV1. The virus encodes an integrase of the fusellovirus-type, which is split after integration into host genome. Due to its morphological similarity to SNDV, APOV1 is suggested to belong to *Guttaviridae* (Mochizuki et al., 2011).

### 1.7.6.3 Coil-shaped viruses

Recently, the first crenarchaeal virus with an ssDNA genome was discovered from Yamagota hot springs in Japan (Mochizuki et al., 2012). *Aeropyrum* coil-shaped virus (ACV) has a circular 24 900 nt positive-sense ssDNA genome. The genome of ACV is the largest viral ssDNA genome known to date. ACV virions are non-enveloped and resemble long cylinders or coils with appendages at both ends (Fig. 4.). The surface of the virion appears to be formed from stacked disks or helix turns. Due to the morphological and genomic uniqueness of ACV, it is suggested to belong to a new viral family called “Spiraviridae” (Mochizuki et al., 2012).

### 1.7.6.4 Bacilliform viruses

*Aeropyrum pernix* bacilliform virus 1 (APBV1) virions are 140 nm long bacilliform rods with one pointed and one round end (Fig. 4.) (Mochizuki et al., 2010). The genome is a circular dsDNA of 5278 bp making it the smallest genome of the known archaeal and bacterial dsDNA viruses. The virus harbors a highly glycosylated MCP, but no appendages such as spikes have been detected on the virion surface (Mochizuki et al., 2010). The virus does not integrate into the host chromosome, but is thought to exist in a carrier state possibly protecting it from the high temperature environment. APBV1 is suggested to represent a new viral family, the *Clavaviridae* (Mochizuki et al., 2010).

### 1.7.6.5 Bottle-shaped viruses

One of the most exceptional virus morphotypes is represented by *Acidianus* bottle-shaped virus (ABV) isolated from an Italian volcanic lake (Häring et al.,
Virions are approximately 230 nm long and resemble bottles with one broad angular and one narrow pointed end (Fig. 4.). Presence of an outer envelope has been observed around ABV virions by TEM. The broad end contains thin filaments that are not involved in the adsorption, which is suggested to occur by the pointed end. This “bottle-neck” structure has been observed to be in a direct contact with the supercoiled nucleoprotein core filament and the viral DNA and presumably contains a channel for DNA transport (Häring et al., 2005b). ABV infects solely “Acidianus convivator” retarding the growth rate, but not causing lysis. The virus has a 23.8 kpb linear dsDNA genome and is currently the only member of the suggested family Ampullaviridae. The virus encodes a protein-primed type B DNA polymerase, which is homologous to DNA polymerases of e.g. bacteriophages PRD1, Φ29, adenoviruses and haloarchaeal viruses His1 and His2, suggesting a close phylogenetic relationship of these replication systems (Peng et al., 2007).

1.8 Viruses of halophilic bacteria

Only a few halophilic bacteriophages, which all represent a head-tailed morphology, have been described prior to this study. However, since the abundance of bacteria in hypersaline environments has been recognized fairly recently (Antón et al., 2000), it is likely that their phages represent a substantial proportion of the viruses in high salt. To date, no phages have been described for the extremely halophilic bacterium Salinibacter ruber, but metagenomic analyses indicate that they might be present in several hypersaline environments (Santos et al., 2010).

The described halophilic bacteriophages infect bacteria from the genera Halomonas, Salicola, and Salinivibrio. A temperate siphovirus, F9-11, was isolated from a lysogenic strain of the moderately halophilic bacterium Halomonas halophila from Spanish salterns. The phage was found to be stable at a wide range (5-25 % w/v) of salinities (Calvo et al., 1988). Another phage, ΦgspC, infecting a strain of Halomonas, is a temperate myovirus with a particularly large genome size (340 kbp). This virus was shown to be stable at slightly elevated temperatures, moderate salinities, and a pH of 6-9, but was sensitive to the absence of salt (Seaman and Day, 2007).

Two halophilic bacteriophages, Salicola tailed phage 1 (SCTP-1) and 2 (SCTP-2), infecting two different strains of Salicola were isolated from solar salterns in Italy representing morphologies of Siphoviridae and Myoviridae...
families, respectively. Both viruses were found to be stable even at saturated salinities (Kukkarro and Bamford, 2009).

Recently, bacteriophage CW02 infecting *Salinivibrio costicola*-like strain SA50 from the Great Salt Lake was isolated and confirmed to belong to the T7-like podoviruses (Shen et al., 2012). The MCP was suggested to occupy the HK97-fold, which is likely present in all head-tailed viruses. CW02 exhibits turret-like appendages on the surface of the virion, which are suggested to aid in the adsorption process and have been described before e.g. for the archaeal haloviruses STIV and SH1 (Shen et al., 2012).

1.9 Virus-host interactions in hypersaline environments

Halovirus research is still at its early stages and as a consequence very little information exists about virus-host interactions in hypersaline environments. As in many other aquatic environments, the high virus abundance in hypersaline environments exceeds the number of prokaryotic cells by at least an order of magnitude (Guixa-Boixareu et al., 1996) and it has been demonstrated that especially the number of spindle-shaped VLPs characteristic to archaeal viruses, increases along with the salinity (Guixa-Boixareu et al., 1996; Oren et al., 1997; Sandaa et al., 2003; Santos et al., 2007). Because cellular eukaryotic predators such as ciliates and flagellates are nearly absent in the highest salinities, viruses have a critical role in the control of halophilic archaeal and bacterial populations (Santos et al., 2007).

1.9.1 Host range of haloviruses

The research on marine bacteriophages has revealed that the different head-tailed virus morphotypes differ also in the host range (Suttle, 2005). Myoviruses have often broad host ranges, siphoviruses intermediate and podoviruses are the most specific to a particular host (Suttle, 2005). Most of the described haloviruses have narrow host ranges although some, such as the myovirus HF1, are able to infect members of different haloarchaeal genera (Tang et al., 2004). Most of the known non-head-tailed haloviruses infect *H. hispanica*, as only SNJ1 infects a strain of *Natrinema* and SH1 also a strain of *Halorubrum* (Porter et al., 2005; Bath et al., 2006; Zhang et al., 2012). Interestingly, no viruses have been obtained for *Haloquadratum walsbyi*, although this archaeum seems to dominate in many different hypersaline environments (Burns et al., 2007). However, several metagenomic analyses point out that these viruses are just
waiting to be discovered. For example, the codon usage and G+C content of an environmental halophage 1 (EHP-1) (existing only as genomic DNA) corresponded to *Hqr. walsbyi* (Santos et al., 2007) and a cluster of viral genomes were found to share CRISPR-spacer sequences (see below) with one strain of this archaeum (Garcia-Heredia et al., 2012).

1.9.2 Antiviral defense mechanisms of haloarchaea

In marine environments, it has been estimated that approximately $10^{23}$ virus infections occur per second (Suttle, 2007). The constant dynamic interactions of viruses and hosts results in hosts having developed different virus defense mechanisms. The recently identified prokaryotic specific adaptive and heritable immune system called clustered regularly interspaced short palindromic repeats (CRISPR) (Barrangou et al., 2007) has also been identified in haloarchaea (Fischer et al., 2012). This is a cluster of repetitive nucleotide sequences that are regularly interspaced by spacer sequences. The spacers can include for example fragments of virus genomes that give the cell resistance to this virus. Archaea can add new spacers and new resistances to CRISPR clusters and so defend themselves against viruses and foreign genetic elements (Marchfelder et al., 2012). Although the CRISPR system has been identified in 90% of the sequenced archaea, it may, however, not be the primary defense system against viruses (Lynch et al., 2012). Other antiviral defense strategies of haloarchaea might be different restriction enzymes and changes in the cell surface structures (Allers and Mevarech, 2005; Cuadros-Orellana et al., 2007; Fischer et al., 2012).

1.9.3 Effect of salt concentration on virus adsorption

Contradictory reports exist about how high salt concentration affects virus adsorption to the host suggesting that the phenomenon greatly depends on the virus (Kukkarao and Bamford, 2009). Many haloviruses lose their infectivity in low NaCl concentration (Kukkarao and Bamford, 2009). In some cases, the increase in salinity results in the change of virus life style, as has been described for Hs1 (Torsvik and Dundas, 1974). At salinities close to saturation, this virus establishes a carrier state and becomes lytic again when the concentration of NaCl is lowered (Torsvik and Dundas, 1980). Another virus, SNJ1 adsorbs to its host only in high NaCl concentrations (Mei et al., 2007). It was observed, that adsorption rate constants can be variable for different viruses suggesting different binding mechanisms to host cells (Kukkarao and Bamford, 2009). No
archaeal virus receptors have been recognized to date, although S-layer proteins and flagella are possible candidates (Porter et al., 2007).

1.9.4 Halovirus life cycles
Although the amount of described haloviruses is low, virus life cycles have been studied for a few cases. Some of the described haloviruses (such as HF1, HF2, and SH1) are virulent exiting their host cell by lysis (Porter et al., 2005; Tang et al., 2004). However, establishment of a carrier state or lysogeny seem to be more common (Stolt and Zillig, 1992; Witte et al., 1997; Porter et al., 2007). It has been suggested, that lysogeny or carrier state could protect viruses from extreme environmental conditions and thus be the most favored life style among extremophilic viruses. When the host becomes subjected to environmental stress such as dilution of brines due to heavy rains, the lytic cycle of temperate haloviruses may be induced (Oren et al., 1997). Some virulent haloviruses on the other hand, require high salinity for maintaining infectivity (Pietilä et al., 2013). The continual virus release without cell lysis, which has been observed for pleomorphic viruses HRPV-1 (Pietilä et al., 2009) and HHPV-1 (Roine et al., 2010) as well as the spindle-shaped His1 (Pietilä et al., 2012), could also be related to virus assembly or to archaeal cell walls that are less rigid than those of bacteria (Porter et al., 2007). Non-lytic life cycles are also common among the studied crenarchaeal viruses (Pina et al., 2011). However, the recently described unique lytic pathway identified for the crenarchaeal viruses STIV and SIRV2 (see above) is challenging the views about non-lytic virus life style dominance in extreme environments (Snyder and Young, 2013). It remains to be seen whether this egress mechanism will apply to haloviruses as well.

1.9.5 Geographic distribution of haloviruses
It has been shown, that viruses can move from one ecosystem to another and thus contribute to lateral gene transfer of microbes. This has been studied e.g. for viruses from marine waters and soil, which were all shown to replicate in marine microorganisms (Sano et al., 2004). Different types of viruses are also known to be transported globally in desert dust most probably adsorbed to soil particles (Griffin, 2007). Extreme environments are often considered to be closed systems where the microbial community has developed without much interaction with the surrounding environment. In acidic hot springs of YNP it was demonstrated that virus movement (e.g. in air) from distant environments controls viral community structure more than mutations (Snyder et al., 2007).
In the same study, it was suggested that there might be little diversity in archaeal extremophilic viruses over wide geographic distances (Snyder et al., 2007).

To date, most of the studies on halovirus diversity in different environments are based on metagenomics and TEM analyses. It seems that regardless of the geographical location or type (thalassohaline or athalassohaline) of the hypersaline environment, the same virus particle morphotypes are routinely observed by TEM with the dominance of the spindles at close to saturated salinities (Guixa-Boixareu et al., 1996; Oren et al., 1997; Sime-Ngando et al., 2010). Metagenomic analyses on the other hand have revealed that viral sequences obtained from different geographically distant hypersaline environments show no hits to public databases, but match to some extent with each other (Santos et al., 2010, 2012). This indicates that there might be similar viruses in different geographical locations. However, since related viruses (such as HRPV-1 and HHPV-1) can have very different genome types (Pietilä et al., 2009; Roine et al., 2010), direct virus isolations on cultivable hosts are needed in order to obtain a more specific understanding about the geographic distribution of haloviruses.
Viruses infecting archaea have been studied only for a few decades and just a small number has been characterized in more detail. These isolates represent virus morphotypes that are known for bacterial and eukaryotic viruses, but there are also virion architectures that seem to be specific for archaeal viruses. Novel virus morphologies have been described especially for crenarchaeal viruses and extreme environments are considered to harbor a range of previously unidentified virus morphotypes. However, it is considered that the limited protein fold space restricts the ways viral capsids can be assembled and therefore it is expected that the number of unique virus morphotypes is drastically lower than the abundance of viruses on the biosphere.

In this study, a wide global sampling of different hypersaline environments was performed in order to isolate new archaeal and bacterial strains and their viruses. The special focus was on finding novel virus morphologies and generally obtaining a better understanding of halovirus structures, host ranges, and life styles. In addition, interactions between all the hosts and viruses in this study were analyzed in order to gain more information about the geographical distribution of haloviruses and their hosts. The second aim was to explore microbial interactions in geographically distant locations. A large set of halophilic archaeal and bacterial strains were explored for the production of halocins. These antimicrobial substances have a short history in the haloarchaeal research and practically no information exists about the halocins of halophilic bacteria.

Among the new isolates, a group of viruses with a novel pleomorphic structure (similar to HRPV-1 and HHPV-1) was chosen for detailed structural and biochemical analysis to explore whether they would form a specific group of related viruses possibly representing a novel structure-based viral lineage.

The aims of this study are specified below:

- to isolate halophilic archaea and bacteria from geographically distant hypersaline environments and to study their antimicrobial agents and interactions especially between isolates that belong to different genera or domain
- to search for new archaeal and bacterial haloviruses and identify novel virus morphotypes focusing on isolates that produce high amounts of infective particles

- to study virus-host interactions and geographic distribution of haloviruses in different globally distant hypersaline environments

- to perform comparative characterization of the life styles and virion structural principles of pleolipoviruses
3 MATERIALS AND METHODS

Salt water and crystal samples used in the study are described in Table S1 (I). The 95 archaeal and bacterial strains used in this study are listed in Table S2 (I). In addition, the archaeal strain *Halorubrum* sp. SS7-4 (III) was used here. Isolated viruses are listed in Table 1 and the used methods are summarized in Table 2. Methods are described in more detail in the original publications.

**Table 2.** Viruses used in this study.

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Virus</th>
<th>Nomenclature</th>
<th>Samplea</th>
<th>Morphotypeb</th>
<th>Isolation hostc</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HRPV-1</td>
<td><em>Halorubrum</em> pleomorphic virus 1</td>
<td>Tra (w)</td>
<td>P</td>
<td><em>Halorubrum</em> sp. PV6 (A)</td>
<td>Pietilä <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>2</td>
<td>SCTP-1</td>
<td><em>Salicola</em> head-tail phage 1</td>
<td>Tra (w)</td>
<td>T (sipho)</td>
<td><em>Salicola</em> sp. PV3 (B)</td>
<td>Kukkaro and Bamford, 2009</td>
</tr>
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<td>3</td>
<td>SCTP-2</td>
<td><em>Salicola</em> head-tail phage 2</td>
<td>Tra (w)</td>
<td>T (myo)</td>
<td><em>Salicola</em> sp. PV4 (B)</td>
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<td>HRTV-2</td>
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<td>T (myo)</td>
<td><em>Halorubrum</em> sp. s1-2 (A)</td>
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<td>T (sipho)</td>
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<td>T (myo)</td>
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<td>Host Code</td>
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<td>T (myo)</td>
<td><em>H. japonica</em> (A)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>HCTV-2</td>
<td><em>Haloarcula californiae</em> head-tail virus 2</td>
<td>SSB (w)</td>
<td>T (spho)</td>
<td>&quot;Har. californiae&quot; (A)</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>HCTV-5</td>
<td><em>Haloarcula californiae</em> head-tail virus 5</td>
<td>SSB (c)</td>
<td>T (spho)</td>
<td>&quot;Har. californiae&quot; (A)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>HVT-2</td>
<td><em>Haloarcula vallismortis</em> head-tail virus 2</td>
<td>SSB (w)</td>
<td>T (spho)</td>
<td><em>H. vallismortis</em> (A)</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>HHTV-2</td>
<td><em>Haloarcula hispanica</em> head-tail virus 2</td>
<td>SSB (w)</td>
<td>T (spho)</td>
<td><em>H. hispanica</em> (A)</td>
<td></td>
</tr>
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<td>HRTV-8</td>
<td>Holarubrum head-tail virus 8</td>
<td>SSB (c)</td>
<td>T (myo)</td>
<td>Holarubrum sp. B2-2 (A)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>HRPV-2</td>
<td>Holarubrum pleomorphic virus 2</td>
<td>SSB (c)</td>
<td>P</td>
<td>Holarubrum sp. SS5-4 (A)</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>HGTV-1</td>
<td>Hologranum head-tail virus 1</td>
<td>SSB (w)</td>
<td>T (myo)</td>
<td>Hologranum sp. SS5-1 (A)</td>
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<tr>
<td>35</td>
<td>HATV-1</td>
<td><em>Haloarcula</em> head-tail virus 1</td>
<td>SSB (c)</td>
<td>T (myo)</td>
<td>*H. sp. SS5-2 (A)</td>
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</tr>
<tr>
<td>36</td>
<td>HRPV-3</td>
<td>Holarubrum pleomorphic virus 3</td>
<td>SP (w)</td>
<td>P</td>
<td>Holarubrum sp. SP3-3 (A)</td>
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<tr>
<td>37</td>
<td>SSIP-1</td>
<td><em>Salisaeta</em> icosahedral virus 1</td>
<td>SP (w)</td>
<td>I</td>
<td>*S. sp. SP9-1 (B)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Virus Code</td>
<td>Host</td>
<td>Length</td>
<td>Virus Type</td>
<td>Description</td>
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<tr>
<td>---</td>
<td>------------</td>
<td>------</td>
<td>--------</td>
<td>------------</td>
<td>-------------</td>
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</tr>
<tr>
<td>38</td>
<td>HSTV-3</td>
<td>Halorubrum sodomense head-tail virus 3</td>
<td>Eil (w)</td>
<td>n.d.</td>
<td>Hrr. sodomense (A)</td>
<td>I</td>
</tr>
<tr>
<td>39</td>
<td>HSTV-2</td>
<td>Halorubrum sodomense head-tail virus 2</td>
<td>Eil (w)</td>
<td>T (myo)</td>
<td>Hrr. sodomense (A)</td>
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</tr>
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<td>40</td>
<td>HRTV-9</td>
<td>Halorubrum head-tail virus 9</td>
<td>Eil (w)</td>
<td>T (myo)</td>
<td>Holorubrum sp. B2-2 (A)</td>
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<td>HRTV-10</td>
<td>Halorubrum head-tail virus 10</td>
<td>Eil (w)</td>
<td>T (myo)</td>
<td>Holorubrum sp. B2-2 (A)</td>
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<tr>
<td>42</td>
<td>HATV-2</td>
<td>Haloarcula head-tail virus 2</td>
<td>Eil (w)</td>
<td>T (myo)</td>
<td>Haloarcula sp. E301-5 (A)</td>
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</tr>
<tr>
<td>43</td>
<td>Eil (w)</td>
<td>n.d.</td>
<td>Halorubrum sp. E301-4 (A)</td>
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<td></td>
<td></td>
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<tr>
<td>44</td>
<td>HTV-1</td>
<td>Halophilic head-tail virus 1</td>
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<td>T (myo)</td>
<td>E200-7</td>
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<td>HRTV-11</td>
<td>Halorubrum head-tail virus 11</td>
<td>SL (c)</td>
<td>T (myo)</td>
<td>Holorubrum sp. SL-5 (A)</td>
<td>I</td>
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<td>HRTV-12</td>
<td>Halorubrum head-tail virus 12</td>
<td>GV (c)</td>
<td>T (myo)</td>
<td>Holorubrum sp. B2-2 (A)</td>
<td>I</td>
</tr>
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<td>47</td>
<td>HGPV-1</td>
<td>Halogeometricum pleomorphic virus 1</td>
<td>CG (c)</td>
<td>P</td>
<td>Halogeometricum sp. CG-9 (A)</td>
<td>I</td>
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<tr>
<td>48</td>
<td>HRPV-6</td>
<td>Halorubrum pleomorphic virus 6 (2008)</td>
<td>SS</td>
<td>P</td>
<td>Halorubrum sp. SS7-4</td>
<td>III</td>
</tr>
</tbody>
</table>

a Tra, Trapani, Sicily, Italy; MdS, Margherita di Savoia, Italy; SSA, Samut Sakhon, Thailand, 2007; SSB, Samut Sakhon, Thailand, 2008; SP, Sedom Ponds, Israel; Eil, Eilat, Israel; SL, Sečovlje, Slovenia; GV, Guardias Viejas, Spain; CG, Cabo de Gata, Spain (w, water sample; c, salt crystal)
b P, pleomorphic, T, head-tail; I, icosahedral; n.d., not determined
c A, archaea; B, bacteria
<table>
<thead>
<tr>
<th><strong>Method</strong></th>
<th><strong>Used in</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Host isolation</td>
<td>I, III</td>
</tr>
<tr>
<td>Virus isolation (direct plating)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Virus isolation (enrichment cultures)</td>
<td>I</td>
</tr>
<tr>
<td>Plaque assay</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Chloroform sensitivity test</td>
<td>I, III</td>
</tr>
<tr>
<td>Virus purification</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Bradford assay for protein concentration measurement</td>
<td>I, III</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate-polyacrylamid gel electrophoresis (SDS-PAGE)</td>
<td>I, III</td>
</tr>
<tr>
<td>Agarose gel electrophoresis</td>
<td>I</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>I, III</td>
</tr>
<tr>
<td>16S rRNA gene sequencing</td>
<td>I, III</td>
</tr>
<tr>
<td>N-terminal sequencing and mass spectrometry</td>
<td>III</td>
</tr>
<tr>
<td>Maximum likelihood phylogenetic analyses</td>
<td>I, II</td>
</tr>
<tr>
<td>Extraction of culture supernatants</td>
<td>II</td>
</tr>
<tr>
<td>Growth curve measurement</td>
<td>II, III</td>
</tr>
<tr>
<td>Agar overlay spot test for detection of antimicrobial activity</td>
<td>II</td>
</tr>
<tr>
<td>Virus host range study</td>
<td>I</td>
</tr>
<tr>
<td>Virus life cycle analysis</td>
<td>III</td>
</tr>
<tr>
<td>Lipid extraction, thin-layer chromatography</td>
<td>III</td>
</tr>
<tr>
<td>Negative stain transmission electron microscopy</td>
<td>I, III</td>
</tr>
<tr>
<td>Electron cryo-microscopy (Cryo-EM)</td>
<td>III</td>
</tr>
<tr>
<td>Electron cryo-tomography (Cryo-ET) and three dimensional modeling</td>
<td>III</td>
</tr>
<tr>
<td>Quantitative biochemical dissociation of virions</td>
<td>III</td>
</tr>
</tbody>
</table>
4 RESULTS AND DISCUSSION

4.1 Global sampling of hypersaline environments for the isolation of halophilic microorganisms

Different types of natural and artificial hypersaline environments represent the primary habitats of halophilic extremophiles and contain remarkably high numbers of haloviruses. Due to the global distribution of hypersaline environments, halophilic microorganisms and their viruses have been studied in several parts of the world, but to date only a handful of halovirus isolates have been specifically characterized (Dyall-Smith et al., 2003; Roine and Oksanen, 2011). There is a lack of comparative analyses on virus-host interactions and the geographic distribution of haloviruses and their hosts. In addition, direct TEM studies of hypersaline waters and virus isolations show contradictory results about the dominant halovirus morphotypes (Guixa-Boixareu et al., 1996; Dyall-Smith et al., 2003; Sime-Ngando et al., 2010). Metagenomic analyses suggest that the isolated haloviruses represent the minority of the viral diversity in nature (Santos et al., 2007). They also indicate that while halovirus communities are spatially diverse (within single environments), genetic similarities have been observed in viral DNA clones obtained from different geographically distant environments indicating world-wide relatedness (Santos et al., 2012). However, culture dependent assays are the only means to provide detailed information about the biology, structures, functions, and host cell interactions of haloviruses. Such studies are currently missing.

In order to isolate new halophilic archaea, bacteria and viruses, we performed a global sampling of nine different hypersaline environments including seven solar salterns (located in Europe, Israel, and Thailand), the Dead Sea (Israel), and the experimental ponds at Sedom (Israel) (See Fig. 1. in I). The obtained samples included both salt water and crystals (See Table S1 in I). Sedom Ponds contain different proportions of the mixed waters of the Dead Sea and the Red Sea. Since the ponds in Sedom have been reconstructed to evaluate the environmental effects of regulating the water level of the Dead Sea with sea water, it is of interest to study whether the microorganisms in this experimental environment relate to those obtained from the Dead Sea or to those from thalassohaline environments. To further expand our archaeal strain set, we added eight culture collection strains into this study (Table 4). Our culture
collection set included *Haloarcula hispanica*, which was originally isolated from Alicante, Spain, and is the host of several known haloviruses with different morphologies (Juez et al., 1986; Roine and Oksanen, 2011). The other seven culture collection strains belong to the family *Halobacteriaceae* and were originally isolated from distant locations including solar salterns and salt lakes (Table 4).

**Table 4. Culture collection strains used in the study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location of isolation</th>
<th>Type of environment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haloarcula hispanica</em> ATCC 33960</td>
<td>Alicante, Spain</td>
<td>Saltern</td>
<td>Juez et al., 1986</td>
</tr>
<tr>
<td><em>Haloarcula marismortui</em> ATCC 43049</td>
<td>the Dead Sea, Israel</td>
<td>Salt lake</td>
<td>Oren et al., 1990; Mylvaganam and Dennis, 1992</td>
</tr>
<tr>
<td><em>Haloarcula quadrata</em> ATCC 700850</td>
<td>Sinai, Egypt</td>
<td>Coastal salt deposit</td>
<td>Oren et al., 1999</td>
</tr>
<tr>
<td>&quot;Haloarcula sinaiiensis&quot; ATCC 33800</td>
<td>Sinai, Egypt</td>
<td>Coastal salt deposit</td>
<td>Javor et al., 1982</td>
</tr>
<tr>
<td><em>Haloarcula vallismortis</em> ATCC 29715</td>
<td>Death Valley, USA</td>
<td>Salt lake</td>
<td>Gonzalez et al., 1979; Torreblanca et al., 1986</td>
</tr>
<tr>
<td><em>Halorubrum sodomense</em> DSM 33755</td>
<td>the Dead Sea, Israel</td>
<td>Salt lake</td>
<td>Oren et al., 1983</td>
</tr>
<tr>
<td>&quot;Haloarcula californiae&quot; ATCC 33799</td>
<td>Baja California, Mexico</td>
<td>Coastal salt deposit</td>
<td>Javor et al., 1982</td>
</tr>
<tr>
<td><em>Haloarcula japonica</em> TR1 ATCC 49778</td>
<td>Noto Peninsula, Japan</td>
<td>Coastal salt deposit</td>
<td>Takashina et al., 1990</td>
</tr>
</tbody>
</table>

Altogether we isolated 61 archaeal and 24 bacterial strains and two halophilic isolates (Table 5). Archaea were obtained from all samples and bacteria from all others except for the saltern of Cabo de Gata, Spain. The halophilic isolates (SP3-1 and E200-7) were mixed cultures that could not be purified despite repeated trials. It is possible that they represent some type of symbiosis, because the two observed colony types in one culture did not grow on their own as a pure culture. Since the number of strains derived from each particular environment was from six to 18 and only one growth temperature and one type of growth media was used, no comprehensive conclusions can be made about the dominant microorganisms in these environments. However, it is probable that these strains represent the most common cultivable members of the halophilic microorganisms that are best suitable for detection of antimicrobial agents and to be used as potential virus hosts.
All the obtained pure cultures of the strains (not including the two halophilic isolates) were subjected to partial 16S rRNA gene sequencing followed by phylogenetic analyses (See Fig. 2. and 3. in I). All the archaeal isolates belonged to the family _Halobacteriaceae_. Although the SHOW group haloarchaea seem to dominate in different hypersaline environments (Burns et al., 2007), we did not isolate any strains related to _Haloquadratum walsbyi_. The dominant archaea in our samples were different strains of _Halorubrum_ (29 strains) that were obtained from all thalassohaline samples as well as from Sedom Ponds. Other strains belonged to _Halogeometricum_ (7 strains), _Haloarcula_ (6 strains), _Halosarcina_ (5 strains), _Halobacterium_ (3 strains), _Halogranum_ (3 strains), _Haloferax_ (2 strains), _Haloplanus_ (1 strain), _Halorhabdus_ (1 strain), _Natronomonas_ (1 strain), and _Salarchaeum_ (1 strain) (I). Previous research highlights the abundance of archaea belonging to either _Halorubrum_ or _Haloarcula_ genera in different, geographically distant hypersaline environments (Oren, 2002b). In terms of species distribution, our saltern samples could be roughly divided into four groups. The Italian salterns of Margherita di Savoia and Trapani, as well as Eilat contained mostly different strains of _Halorubrum_ and _Haloarcula_. The Spanish salterns were characterized by several isolates from the genera _Halogeometricum_ and _Halosarcina_. Samut Sakhon represented a mixture of these two environments and the saltern of Sečovlje contained mostly strains of _Halobacterium_ which were not obtained from any other samples. The archaeal strains from Sedom Ponds belonged to _Halorubrum_ and _Haloferax_, but there was also one _Haloplanus_ isolate. Members of this genus have previously been characterized from this particular environment and from salterns (Bardavid et al., 2007; Cui et al., 2010). Only one archaeal strain belonging to _Halorhabdus_ was obtained from the Dead Sea. The strain was closely related to _Halorhabdus utahiensis_, originally isolated from the Great Salt Lake of Utah (Waino et al., 2000). These results indicate that although archaea from _Halobacteriaceae_ are globally detected in different salterns, there might be some spatial trends in the dominant species distribution (I).

The number of bacterial strains (24) isolated from our samples corresponds to 28% of the total number of strains (87). This number seems fairly high and similar proportions have been described before e.g. from the salterns of Alicante, Spain (Antón et al., 2000). No strains resembling the extremely halophilic bacterium, _Salinibacter ruber_ (Antón et al., 2002), were obtained in our study, but based on the partial 16S rRNA gene sequences, our two _Salisaeta_ sp. isolates (SP9-1 and SP10-4) from Sedom Ponds were closely related to _S. ruber_ and
Salisaeta longa. S. longa has previously been described from the same environment (Vaisman and Oren, 2009). Apart from S. ruber, strains of Salisaeta are moderately halophilic (Vaisman and Oren, 2009). Strains of Salicola, Halomonas, and Pontibacillus were isolated from the Italian salterns, Eilat, Sedom Ponds and Sečovlje. In addition, two strains belonging to the anaerobic genus Rhodovibrio were isolated from Guardas Viejas but not from other samples. From the Dead Sea, several strains of Chromohalobacter were obtained. Strains of this bacterium have commonly been described from the Dead Sea (Ventosa et al., 1989). It seems evident that halophilic bacteria represent a significant proportion of the microbiota in hypersaline environments and can be isolated by the culture-dependent approach (I).

Although most of the samples were from solar salterns and only two from other types of hypersaline environments, the strain sets derived from thalassohaline, athalassohaline, and mixed environments differed from each other. The strains from the Dead Sea were not closely related to those derived from thalassohaline environments. Sedom Ponds samples also contained archaea and bacteria that were not detected in the other samples. At least according to the isolation of halophilic strains, it seems that the Sedom Ponds environment is drastically different from the Dead Sea and the mixing of the Red Sea water with the Dead Sea might affect microbial diversity in the Dead Sea (I).

Table 5. Summary of the isolated archaeal and bacterial strains, halocin producers and sensitive strains, and viruses.

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of samples</th>
<th>Type</th>
<th>Archaeal isolates</th>
<th>Bacterial isolates</th>
<th>Halophilic isolates</th>
<th>Halocin producers</th>
<th>Halocin sensitive strains</th>
<th>Archaeal viruses</th>
<th>Bacteriophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Margherita di Savoia 4 Saltern (thalassohaline)</td>
<td>9 3 5 6 20</td>
<td>1</td>
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<td></td>
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<tr>
<td>Trapani 2 Saltern (thalassohaline)</td>
<td>3 4 3 2</td>
<td>1 2</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Eilat 4 Saltern (thalassohaline)</td>
<td>11 3 1 6 11 9</td>
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<td></td>
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<td></td>
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<tr>
<td>Sedom Ponds 4 Experimental ponds (Mixed)</td>
<td>4 3 1 4 5 1</td>
<td>1 1</td>
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<tr>
<td>Dead Sea 1 Salt lake (thalassohaline)</td>
<td>1 5 2</td>
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</tr>
<tr>
<td>Guardias Viejas 1 Saltern* (thalassohaline)</td>
<td>6 2 7 1</td>
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<td></td>
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<tr>
<td>Cabo de Gata 1 Saltern (thalassohaline)</td>
<td>7 4 6 1</td>
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</tr>
<tr>
<td>Secovlje 1 Saltern (thalassohaline)</td>
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<td></td>
</tr>
<tr>
<td>Samut Sakhon 5 Saltern (thalassohaline)</td>
<td>14 3 10 13 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Culture collection 8</td>
<td>1 7</td>
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<td>Total 23 3 69 24 2 36 65 45 4</td>
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<td></td>
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</table>
4.2 Production of antimicrobial substances (halocins) among halophilic archaea and bacteria

To date, all the described halocins are produced by archaea of *Halobacteriaceae* and practically no reports exist about antimicrobial agents of halophilic bacteria (O'Connor and Shand, 2002). In order to study halocin production among halophilic archaea and bacteria in different types of hypersaline environments, 82 archaeal and bacterial strains obtained from the global sampling (I) were chosen for the halocin study (II). In addition, the eight culture collection strains were included in the study (Table 4). For most of the studied halocin producers, halocin production is highest at the transition from exponential to stationary phase (O'Connor and Shand, 2002). The culture supernatants of the tested strains were collected at the early stationary phase and applied on the lawns of indicator strains, which were at the exponential growth phase at the time of plating. After testing all the strains against all, 27 archaeal and nine bacterial strains were identified as halocin producers (Table 5; II). Although previously, some strains have been reported being sensitive to their own halocins (Torreblanca et al., 1994), this was not observed in our study (II). Archaeal producers were distributed in eight genera, *Halorubrum*, *Haloarcula*, *Haloplanus*, *Halogramnum*, *Haloferax*, *Halosarcina*, and *Halogeometricum*. The producers of the best characterized halocins belong to genera *Haloferax* and *Halobacterium* (Shand and Levy, 2007). Two strains of *Haloferax* included in this study were found to produce halocins with exceptionally broad inhibition spectrum (See Table 2 in II). Especially *Haloferax* sp. s5a-1 culture supernatant was able to inhibit 58 other strains, which was more than four times the number observed for *Halorubrum* sp. E200-4 supernatant, which inhibited 12 strains (See Table 2 in II). Interestingly, the *Haloferax* strains were not sensitive to any halocins in the study. Our *Halobacterium* strains were not detected to produce any halocins, but were sensitive to halocins produced by strain *Haloferax* sp. s5a-1. In addition, sensitivity to halocins was observed for archaea in the genera *Halorubrum*, *Haloarcula*, *Halogramnum*, *Salarchaeum*, *Natronomonas*, *Halosarcina*, and *Halogeometricum* (II).

Bacterial halocins producers were detected in four genera, *Halomonas*, *Salicola*, *Salinivibrio*, and *Pontibacillus* (II). Bacterial producers did not exhibit broad inhibitory spectra as they inhibited one or two sensitive strains. Halocin sensitive strains were observed in five genera, *Chromohalobacter*, *Halomonas*,
Rhodovibrio, Salisaeta, and Pontibacillus. The last four genera were inhibited by archaeal halocins as well.

The most remarkable observation among the halocin producer-sensitivity interactions was the high number of halocins able to inhibit the growth of strains belonging to different genera and sometimes even different domain (See Fig. 2. in II). Archaea that produced halocins inhibiting bacteria belonged to the genera Halorubrum, Haloferax, and Halogranum. Although to date, no halocins of halophilic bacteria have been described in detail, there are reports about a case study in Utah, were extreme halophiles from all three domains of life including haloarchaea, halophilic bacteria, and halophilic fungi, were found to inhibit each other (Shand and Levy, 2008). However, no detailed information exists about these antimicrobial agents. Archaeal halocins have also been tested on pathogenic bacteria and surprisingly some were able to inhibit these strains (Kavitha et al., 2011). Usually bacteriocins are known to inhibit closely related strains (apart from lactococcal antimicrobials, such as nisin, which also has a broad inhibitory spectrum (De Vuyst and Leroy, 2007)), but at least here, halocins of bacterial producers were able to inhibit members of different genera. In addition, one producer strain, Salicola sp. s3-1, was shown to inhibit the growth of an archaeal strain, Halorubrum sp. s1-2. The broad inhibitory properties of many halocins might be suitable for an extreme environment, where species diversity is relatively low.

4.3 Haloviruses infecting haloarchaea and halophilic bacteria isolated from hypersaline samples

In order to expand the number of characterized haloviruses, and to explore their morphological diversity, samples from nine different geographical regions were studied for haloviruses (See Fig. 1. and Table S1 in I). Viruses were screened for 95 hosts isolated from the same samples (including the 87 isolated strains and eight culture collection strains (See Table S2 in I). Altogether 49 viruses, 45 infecting archaea, and four infecting bacteria, were isolated (Table 5; I). From these viruses seven isolates, HRPV-1, HHPV-1, SCTP-1, SCTP-2, HHTV-1, HCTV-1, and HRTV-1, have been described in previous studies (Kukkaro and Bamford, 2009; Pietilä et al., 2009; Roine et al., 2010). In addition to salt water samples, viruses were also obtained from salt crystals. From the 42 new virus isolates, nine were isolated from crystals (See Table 1 in I). In previous research, only halophilic archaea have been characterized from salt crystals (Fendrihan et
al., 2006), but this study shows that also their viruses can be recovered from dissolved crystals.

The original isolation hosts of the archaeal viruses belonged to four genera of Halobacteriaceae: Halorubrum (23 viruses), Haloarcula (19 viruses), Halogeometricum (one virus), and Halogranum (one virus) (I). Viruses infecting Halogeometricum or Halogranum have not been described before. In addition, one virus was described for the halophilic isolate E200-7. The isolated halophilic bacteriophages infected Salicola (three phages) or Salisaeta (one phage).

Viruses were isolated both by direct plating of the water samples on host lawns, or by enriching the sample with the host culture suspension. Approximately one third of the viruses were isolated from enrichment cultures (I). The second method is suitable for detecting viruses that might be less abundant in the samples. Several different plaque morphologies were observed on host lawns. These included different sizes of clear plaques, clear plaques with hazy edges, and hazy plaques. All unique plaques were isolated and purified three consecutive times after which plate lysates were prepared. From the new virus isolates, 25 produced high titer of infective particles (>10¹⁰ pfu ml⁻¹). The rest had titers ranging from 10⁵ to 10⁹ pfu ml⁻¹ (See Table 1 in I)

4.4 Different virus morphotypes observed among the new isolates

Only the isolates with a lysate titer above 10⁷ pfu ml⁻¹ were chosen for morphological characterization. From our isolates, 47 had a lysate titer above this, but only 38 of them were successfully purified by rate-zonal and equilibrium centrifugation (I). Viral structural proteins were analyzed by Tricine-sodium dodecyl sulphate polyacrylamid gel electrophoresis (Tricine-SDS-PAGE). The protein pattern as well as the host range and morphology were used as a criterion for uniqueness of the isolates.

The morphology of the purified viruses was studied by negative stain TEM. Based on this analysis, 38 viruses belonged to five different virus morphotypes (Fig. 5.). Virion diameters of all virus isolates ranged from approximately 50-110 nm (I). Head-tailed viruses represented the majority (82 %) of the 38 isolates (Fig. 5.). All the three types of head-tailed viruses of the viral order Caudovirales were observed. Myoviruses were the most common followed by siphoviruses and
only one isolate had podovirus morphology. This isolate, HSTV-1, is the first podovirus described infecting archaean.

Icosahedral non-tailed viruses represented rare morphotypes as only two isolates, the archaeal virus HHV-2 and the bacteriophage SSIP-1, had this morphotype (Fig. 5.; I). SSIP-1 infecting Salisaeta bacterium is the first described icosahedral, non-tailed bacteriophage infecting a halophilic host. Both the virus and its host were isolated from Sedom Ponds. Icosahedral non-tailed viruses are extremely rare among bacteria, as only 4% of all known bacteriophages are not head-tailed viruses (Ackermann and Prangishvili, 2012).

In addition to the icosahedral tailles and head-tailed viruses, one novel, pleomorphic virus morphotype was detected among the new isolates. Pleomorphic viruses represented 13% of the isolates, indicating that this type of viruses is relatively common in hypersaline environments (Fig. 5.; I). Altogether five viruses, including HRPV-1 (Pietilä et al., 2009), HHPV-1 (Roine et al.; 2010), HRPV-2, HRPV-3, and HGPV-1 (Table 2; I), had pleomorphic morphology. The detailed characterization of HRPV-1 and HHPV-1 has revealed that these viruses resemble a membrane vesicle and lack a nucleocapsid. From previous research, only bacteriophages L2 and L172 infecting Acholeplasma laidlawii (Dybvig et al., 1985) resemble the pleomorphic viruses isolated here.

Plaque assay has been considered to favor the isolation of lytic viruses (Sime-Ngando et al., 2010). All the known head-tailed viruses of both archaean and bacteria are either virulent or temperate and recognizable using plaque assay (Ackermann and Prangishvili, 2012). In our study, there were some features connecting virion morphology and the plaque morphology. Head-tailed virus plaques were often clear or clear with hazy edges (halos). The clear plaques are produced by virulent viruses, whereas the appearance of halos around clear plaques (of head-tailed phages) is known for T-even phages and results from lysis inhibition (only a fraction of hosts are lysed during infection) (Condit, 2007). These two plaque morphologies were characteristic also to our head-tailed viruses (I). The plaques produced by the two icosahedral viruses were clear and medium-sized indicating that these viruses are probably virulent. The plaques produced by pleomorphic viruses were hazy, indicating non-lytic lifestyle. The isolation of five pleomorphic viruses suggests that also non-lytic viruses could be detected using plaque assay.
Figure 5. Schematic illustration of the morphotypes of the isolated viruses. (a) from left to right: head-tailed myo-, sipho-, and podovirus (b) pleolipovirus (c) icosahedral virus (d) the percentage of different virus morphotypes of the isolated viruses

4.5 Virus host range

Host range of the new haloviruses was studied by cross-testing all the 49 viruses against all the 95 hosts (I). As expected, archaeal viruses did not infect bacteria or vice versa. It was shown that virus morphology to some extent correlated with the scale of the host range (broad or narrow). Especially in the case of head-tailed viruses, morphology and host range followed similar pattern which has previously been observed for marine bacteriophages (Suttle, 2005) (see Introduction). Myoviruses had the broadest host ranges and often infected strains from different genera (I). However, archaea most often infected by myoviruses belonged to the genus *Halorubrum*. Siphovirus host ranges were intermediate, but usually ranged only to strains of the same genus, most often *Haloarcula*. The podovirus isolate was very specific to its host, “Har. sinaiensis”. From all the isolated viruses, myoviruses were the most common head-tailed morphotype followed by siphoviruses and podoviruses (See Fig. 4. in I). Myo- and siphoviruses are also most commonly isolated for marine bacteria, although podoviruses have been observed to be most common by TEM analyses (Suttle, 2005). This is probably associated with the host range, as it is easier to isolate viruses with broad host ranges.
The non-head-tailed virus isolates had clearly narrower host ranges than the head-tailed viruses (See Table S3 in I). The icosahedral virus HHIV-2 infected the strain *Haloarcula* sp. PV7, in addition to its isolation host, *Har. hispanica*. The icosahedral bacteriophage SSIP-1 was specific to its host *Salisaeta* sp. SP9-1, even thought it was later tested with different strains of the same genus as well as with *Salinibacter ruber* (Aalto et al., 2012). All the pleomorphic isolates infected only their isolation hosts, that belonged to the genera *Halorubrum* (three viruses), *Haloarcula* (one virus), and *Halogeometricum* (one virus).

### 4.6 Geographical distribution of haloviruses and halocins in hypersaline environments

Since only a limited amount of organisms can survive in extreme environments, it could be assumed that these environments have similar features around the world. One of the most interesting aspects of the global sampling project of hypersaline environments was to study the antagonistic interactions of the obtained halophilic microorganisms as well as their sensitivity to viruses (I; II). We tested all the hosts used in the halocin assay against each other for antimicrobial interactions and all the viruses against the available halophilic archaea and bacteria. In both cases, a wide network of geographically distant interactions was observed (Fig. 6.).
Figure 6. Virus-host interations and halocin production and sensitivity of archaeal and bacterial strains. The large squares represent the isolation locations marked with their specific colors (See I, Fig. 1.). The culture collection strains are grouped together (black). Each location box is filled with white rectangles with curved ends (archaea) and angular ends (bacteria). For strain numbering see (Table 1 in II). The colored circles indicate archaeal viruses and squares bacteriophages. Active halocins are indicated by triangles pointing upwards (archaeal producers) or downwards (bacterial producers). Viruses are numbered according to Table 2. Halocin numbering is the same as for the archaeal and bacterial strains shown here. The strains without numbers were not used in the antimicrobial assay but were used as virus “baits”.

In the case of haloviruses, 140 virus-host interactions were detected and several strains that in the initial screen were not recognized as virus hosts were infected by viruses from geographically distant locations (Fig. 6; I). As a matter of fact, from the 140 interactions, 100 were detected between viruses and hosts from distant locations and only 40 within the same sampling site (Fig. 6.). The highest number of geographical virus-host interactions was observed between viruses from Margherita di Savoia, Eilat, Samut Sakhon and in the culture collection strain group (Fig. 6.; I). Only the Dead Sea and the saltern of Cabo de Gata did not have any such interactions. In Guardias Viejas, Sečovlje, and Sedom Ponds, the interactions were observed for certain Halorubrum strains only. Since the head-tailed viruses had the broadest host ranges, the majority of the interactions were due to viruses with this morphotype. The wide host range of these viruses might be the reason for their frequent isolations in addition to lytic life styles. The viruses with other morphotypes seem clearly more host-specific. If the described VLPs in hypersaline samples represent infective viruses (Guixa-Boixareu et al., 1996; Sime-Ngando et al., 2010), it is possible that they infect hosts that have not yet been cultivated.

Interestingly, the majority of the halocin production-sensitivity interactions were observed in locations where virus-host interactions were nearly absent (Fig. 6.; II). Especially in Sedom Ponds and Guardias Viejas a large number of halocin sensitive strains were detected. In Sedom Ponds, the producers originated from seven different locations. Even the strains of the Dead Sea were the targets of halocin producers (Fig. 6.). However, no specific trends could be observed comparing the virus-host interactions and the halocin producer-sensitivity interactions. It was, however, noted that such strains which were attacked by several different viruses, were often also targets of different halocin producers. These strains included e.g. Halorubrum sp. SS1-3, Haloarcula vallismortis, Halorubrum sodomense, and Halorubrum sp. GV-9 (Fig. 6.). Since it is
probable that all strains are infected by viruses and most strains also produce halocins, an interesting aspect is whether halocins can somehow affect virus infectivity or life cycle. Very few studies exist about this subject. Bacteriocins produced by enterococci have been shown to have antiviral activity against Herpes simplex viruses 1 and 2 and lactococcal bacteriocins might induce the lytic cycle of prophages in the lysogenic strains (Wachsman et al., 2003; Madera et al., 2009). In addition, some bacteriocins morphologically resemble head-tailed virus tails and a common origin of these components has been suggested (Veesler and Cambillau, 2011).

It has been considered that the production of halocins would result from competition of nutrients and life space between different organisms sharing the same environment (Torreblanca et al., 1994). However, antimicrobial interactions might also increase species diversity. This hypothesis proposed that the producer strain has an advantage over the sensitive one, the resistant strain can overcome the producer, and the halocin-sensitive strain competes with the resistant one (Kirkup and Riley, 2004). Also viruses affect species diversity. Kill the winner -model described for marine viruses suggests that viruses attack the dominant microorganisms thus indirectly preserving the less dominant species (Thingstad and Lignell, 1997; Hoffmann et al., 2005).

### 4.7 Isolation of pleolipoviruses

One of the major aims of the global virus hunt was to find novel virus morphotypes and explore whether the wide range of different morphologies observed for crenarchaeal viruses would also apply for haloviruses. While most of the isolated viruses represented well-known morphologies, we have detected one new virus morphotype portrayed by HRPV-1 and HHPV-1 (Pietilä et al., 2009; Roine et al., 2010), as we also have isolated three additional viruses (HRPV-2, HRPV-3, HGPV-1) with the same morphology (I). In addition to the global sampling project (I), we performed another search for viruses and hosts from Samut Sakhon and isolated a new virus (HRPV-6) with pleomorphic morphology infecting a *Halorubrum* strain (*Halorubrum* sp. SS7-4) (III). In addition, His2, which was previously described as a spindle-shaped virus, but shown to have sequence similarity to HRPV-1 and HHPV-1 (Senčilo et al., 2012), was included in the comparative study, and confirmed to be pleomorphic (III). Since the pleomorphic isolates were detected in five different locations (See

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60
Table 1 in III), we can assume that these viruses are globally distributed and common for archaeal extreme halophiles.

The protein profiles of the purified pleomorphic virions were studied by Tricine-SDS-PAGE and confirmed to be similar consisting of one (or two for His2) large major structural protein of 50-79 kDa and one (or two for HGPV-1) small major structural protein of 7-14 kDa (Fig 7; See Fig 3B in III). N-terminal sequencing as well as biochemical dissociation was applied to analyze virion structural proteins (VP) and identify genes encoding them. Virion proteins were named according to corresponding genes (Senčilo et al., 2012).

Thin-layer chromatographic analyses revealed that all the pleomorphic viruses contained lipids which were unselectively acquired from the host (III). Lipid pattern of His2 was identical to its host *Har. hispanica*. The other viruses showed minor deviations from the host lipid composition (III).

Virion quantitative dissociation analysis was performed for all viruses except for HRPV-1, for which it had been done previously and which was used here for comparison (Pietilä et al., 2010). For all viruses, proteinase K treatment in high salinity digested the large major structural protein, which confirmed it to be the spike protein (Fig. 5; III). When proteinase K treated virions were exposed to low salinity, the genomes were released, while the small major structural protein remained associated with the membrane vesicle indicating that it is an internal membrane protein (See Fig. 5. in III). This was shown previously for HRPV-1 (Pietilä et al., 2010). Minor differences in the biochemical dissociation were observed among the different viruses, e.g. HRPV-2 and HRPV-6 genomes remained associated with the membrane vesicle at lowered salinity and were fully or partially released only when further treated with proteinase K. Based on their morphology and genetic and biochemical properties, these viruses were collectively named as pleolipoviruses.

4.7.1 Pleolipovirus life cycles

The life cycles of HRPV-1 and HHPV-1 have been confirmed to be non-lytic only slightly retarding host growth (Pietilä et al., 2009; Roine et al., 2010). His2 on the other hand, had been previously characterized as a lytic virus (Bath et al., 2006). In order to study whether the group of pleomorphic viruses shares the same life style, we performed life cycle studies of all the viruses except HRPV-1 and HHPV-1 (III). The exponentially growing hosts were infected with a high multiplicity of infection (MOI 15) and the turbidity of the infected and non-infected cultures as well as the number of plaque forming units (PFU) was
monitored for 29 h. None of the viruses caused lysis of the host cells as the turbidity of the culture was not reduced significantly, and the amount of viable cells was relatively high in the infected cultures (See Fig. 1. in III). Instead viruses were released continuously with high titers indicating persistent, non-lytic infection similar to what was described for HRPV-1 and HHPV-1 (Pietilä et al., 2009; Roine et al., 2010). There were, however, slight differences in the growth rates of the host cells infected by different viruses. His2 retarded host growth more than the other viruses, whilst for HGPV-1 hardly any retardation could be detected and the amount of viable cells was similar in infected and non-infected cultures (III, Fig 1).

The non-lytic life style of pleolipoviruses resulted in typical hazy plaque morphology, which was similar for all the seven viruses (III). The observed hazy plaques most probably indicate host growth retardation with no lysis. The presence of lipids that are non-selectively acquired from the host suggests that pleolipoviruses exit the host cells by a budding mechanism as has been postulated for HRPV-1 and HHPV-1 (Pietilä et al., 2010; Roine et al., 2010).

<table>
<thead>
<tr>
<th>Virus</th>
<th>HRPV-1</th>
<th>HRPV-2</th>
<th>HRPV-3</th>
<th>HRPV-6</th>
<th>HHPV-1</th>
<th>HGPV-1</th>
<th>His2</th>
<th>kDa</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Virion diameter (nm)</td>
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<td>54</td>
<td>67</td>
<td>49</td>
<td>52</td>
<td>56</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Genome type</td>
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<td>ssDNA circular</td>
<td>dsDNA circular</td>
<td>ssDNA circular</td>
<td>dsDNA circular</td>
<td>dsDNA circular</td>
<td>dsDNA linear</td>
<td></td>
</tr>
<tr>
<td>Genome size (bp/nt)</td>
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<td>10050</td>
<td>6770</td>
<td>0549</td>
<td>0002</td>
<td>9694</td>
<td>10007</td>
<td></td>
</tr>
<tr>
<td>Host</td>
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<td>Halorubrum sp. 959-4</td>
<td>Halorubrum sp. SP3-3</td>
<td>Halorubrum sp. 957-4</td>
<td>Haloarcana arispamica</td>
<td>Haloarcula arispamica</td>
<td>Haloarcula arispamica</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 7.** General characteristics of pleolipoviruses. The side of the EM-square represents 100 nm. Protein profile of each virus on tricine SDS-PAGE is shown on the right. Protein standard masses are shown far right. The lowest bands indicate viral lipids. TEM micrographs of HRPV-1 and HHPV-1 are courtesy of M.K. Pietilä and E. Roine, respectively.

### 4.7.2 Morphological characterization of pleolipovirus architecture

Because the negative stains used in TEM analyses (ammonium molybdate and uranyl acetate) resulted in different particle morphologies and in some cases loss
of infectivity, cryo-EM and electron cryo-tomography (cryo-ET) were applied to study the pleomorphic virion architecture (III). Cryo-EM analyses revealed that all the isolates had similar virus morphology characterized by roughly spherical virions with particle diameters of approximately 41-71 nm decorated by spikes (See Fig 3A in III). The pleomorphicity of the virions was confirmed by sedimentation analyses and cryo-ET. During rate-zonal centrifugation, virus particles sedimented as a wide light-scattering band. Because virus infectivity was high throughout the band accompanied by similar ratio of the two major structural proteins, individual virions seem to have different sedimentation coefficients indicating pleomorphicity (See Fig. 4. in III). Cryo-ET of HRPV-1 virions revealed that the particles were covered by club-shaped spikes that were irregularly distributed along the virus membrane surface (See Fig 6 in III).

The structural analyses conclude that pleolipoviruses have a simple architecture consisting of a spike-decorated membrane vesicle, which encloses the viral genome without associated nucleoproteins (III). In this study, the structure of all the seven isolates was shown to be similar with small size variation (Fig. 7.). The genome analyses of HRPV-1 and HHPV-1 revealed that these related viruses with similar morphology contained different genome types (either ssDNA or dsDNA) (Fig. 7.) (Pietilä et al., 2010; Roine et al., 2010; Senčilo et al., 2012). All the seven viruses were shown to be related to each other by gene synteny as they were found to share a cluster of five conserved genes (including the genes for the spike and internal membrane proteins) (Senčilo et al., 2012). In addition, similarities were observed within the pleolipovirus genomes and putative proviral regions in the genomes of several sequenced haloarchaea (Senčilo et al., 2012). Although the genomes share sequence similarity and gene synteny, the genome types were different. Pleolipovirus genomes can be either circular ssDNA (HRPV-1, HRPV-2, HRPV-6), circular dsDNA (HRPV-3, HGPV-1), or linear dsDNA (His2) (Fig. 7.) (Bath et al., 2006; Senčilo et al., 2012).

These biological and structural studies along with the genome analysis have revealed that the seven characterized pleolipoviruses form a unique group of related viruses with different genome types (III). To date viruses are classified according to their genome type and replication properties, and no group of related viruses with different nucleic acid types is known (King et al., 2012). The identification of pleolipoviruses thus challenges the current classification of viruses by International Committee on Taxonomy of Viruses (ICTV). The bacterial plasmaviruses L2 and L172 infecting Acholeplasma laidlawii might be...
similar to pleolipoviruses (Dybvig et al., 1985). These viruses have non-lytic life styles, similar protein profiles and virion architecture (Dybvig et al., 1985). It seems that the haloarchaeal pleolipoviruses and the bacterial plasmaviruses might form a distinct structure-based viral lineage indicating common ancestry. The described pleomorphic viruses infecting eukaryotes (e.g. paramyxoviruses, herpesviruses, influenzaviruses and lentiviruses) do not resemble pleolipoviruses as they have nucleoprotein particles inside the external envelope (King et al., 2012). Interestingly, pleolipovirus-like membrane vesicles associated with cellular DNA have been described for euryarchaea from the order Thermococcales (Soler et al., 2008). These might be either previous pleomorphic viruses or structures that have evolved to viruses.
5 CONCLUSIONS AND FUTURE PERSPECTIVES

This work presents the most extensive culture-dependent study on halophilic microorganisms, their viruses and antimicrobial agents that has been done to date. The characterization of over 80 archaeal and bacterial strains and 42 new viruses doubled the number of described archaeal viruses and viruses of halophilic bacteria. As a consequence, this work has influenced several new research projects emphasizing on archaeal viruses.

One of these works has been the genomic comparison of archaeal head-tailed myo- and siphoviruses (Senčilo et al., 2013), utilizing viruses described here (I). The study presents the complete genome sequences of four myoviruses (HGTV-1, HRTV-5, HRTV-7 and HRTV-8) and six siphoviruses (HCTV-1, HCTV-2, HCTV-5, HHTV-1, HHTV-2 and HRTV-4) and shows that viruses HRTV-5 and HRTV-8 are related to each other and the previously described myoviruses HF1 and HF2. The genomes of head-tailed archaeal viruses seem to be mosaics, like the head-tailed bacteriophages (Senčilo et al., 2013). In addition, structural and assembly proteins similar to those observed for head-tailed bacteriophages were identified supporting the hypothesis that these viruses have a common origin (Senčilo et al., 2013). This hypothesis was further supported by Pietilä et al., who described the life styles, genome sequences and the detailed capsid structures of the myovirus HSTV-2 and siphovirus HVTV-1 also isolated here, using cryo-EM and three-dimensional image reconstruction (Pietilä et al., 2013). It was proposed that the maturation of HVTV-1 is similar to the coliphage HK97, which is the type member of the structure-based viral lineage of HK97-like viruses (Pietilä et al., 2013). In addition, HSTV-2 genome was shown to be related to HF1 and HF2 (Pietilä et al., 2013). These viruses together with HRTV-5 and HRTV-8 originate from geographically distant environments but are still related supporting the global distribution of viruses and their movement (Snyder et al., 2007).

The isolated icosahedral viruses, the archaeal virus HHIV-2 and the halophilic bacteriophage SSIP-1 (I) have been further studied and both were revealed to belong to the less-studied subgroup (those with two MCPs) of PRD1-like viruses represented by e.g. SH1 and Thermus thermophilus phage P23-77 (Aalto et al., 2012; Jaakkola et al., 2012). Like SH1, HHIV-2 was confirmed to be a virulent virus with similar protein pattern and genome synteny with SH1 (Jaakkola et al., 2012). However, despite infecting the same host, HHIV-2 genes
encoding receptor recognition vertex were different than for SH1 (Jaakkola et al., 2012). SSIP-1 was found to be a lytic virus encoding 57 ORFs of which 38 are unknown in databases (Aalto et al., 2012). The virus acquires its lipids selectively from the host and the infectivity highly depends on the exponential growth of the hosts as well as high salinity (Aalto et al., 2012). Cryo-EM analyses revealed that the T=49 icosahedral capsid of SSIP-1 encloses an internal lipid membrane harboring the dsDNA genome (Aalto et al., 2012).

While the majority of the isolated viruses represented well-known universal morphologies supporting the hypothesis of limited abundance of virus architectures (see Aims of the study), the isolation of pleolipoviruses brought us one example of novel virus architecture (I; III). The simultaneously performed genomic study revealed that these globally distributed related viruses have very different genome types (Senčilo et al., 2012), which challenges the current classification of viruses. In the future, the most interesting aspect will be to study the entry and egress mechanisms of pleolipoviruses. It is considered that the spike proteins are responsible for fusion of the viral and cellular membranes, but to date nothing is known about fusion in archaeal viruses.

The search for antimicrobial agents led us into characterization of a high number of halocin producing strains, which were identified at the genus level (I, II). This represents the first detailed report of cross-domain inhibition of halocins as well as introduces halocins produced by halophilic bacteria. The study will be continued in the future by specific characterization of some of the halocins, which involves e.g. purification of halocin proteins. Most probably the halocins from producers with broad inhibitory properties will be chosen for these further studies.

Following this global survey, several new samplings have been performed in Samut Sakhon, Thailand during three consecutive years (Atanasova, unpublished data). These samples will be studied for haloviruses and their hosts focusing on spatial and temporal diversity of virus-host interactions.

Microbial interactions in hypersaline environments were found to be geographically distributed and antimicrobial agents were affecting organisms from two different domains (II). As a consequence, we now have advanced considerably our knowledge of haloarchaea, halophilic bacteria, their viruses and halocins in different hypersaline environments. However, we still do not know the extent of interactions between organisms from the three domains of life and their viruses (and antimicrobials) in such environments. For example, no viruses have been characterized for the primary producer *Dunaliella* (Oren et al., 1997)
residing in high salinity. However, different types of world-wide distributed green microalgae are known to be infected by large dsDNA viruses belonging to *Phycodnaviridae* (Wilson et al., 2009). Recently, the studies of an Antarctic hypersaline lake (Organic Lake) have revealed that these viruses are preyed by virophages reflecting the diverse interactions between viruses and microorganisms in extreme environments (Yau et al., 2011). The high number of virus-host interactions between spatially distant environments described here are in line with the observations about virus movement between geographically isolated environments (Snyder et al., 2007).

Overall the results of the current work support the viral lineage hypothesis that viruses can be grouped into a low number of virion architecture-based lineages due to limited protein fold space (Bamford, 2003; Abrescia et al., 2012). Only one new virus type was obtained using this random sampling the rest being already known morphotypes. This indicates a strong limitation of available virion structures.
ACKNOWLEDGEMENTS

This work was performed at the Academy of Finland Centre of Excellence in Virus Research (2006-2011) and Programme on Molecular Virology led by Academy Professor Dennis Bamford, at the Institute of Biotechnology and Department of Biosciences, Faculty of Biological and Environmental Sciences, University of Helsinki, under the supervision of Docent Hanna Oksanen.

I wish to express my most sincere gratitude to Hanna, for your excellent supervising throughout this work. You were always there with a fast and convenient solution to any challenge on the way and introduced me into scientific literature, methods, and opportunities to learn more. Thank you also for treating us (your students) so fairly and equally and making sure that no valuable details have been neglected in the finished works. I really enjoyed doing this work.

I owe my deepest gratitude to Dennis for giving me the chance to join the Programme on Molecular Virology, which provided an excellent environment for this work. Thank you for always having time to equally advice and support each one of us, no matter how busy your schedule was. Thank you for letting us lean on your long experience and scientific expertise, creativity and inventiveness throughout the years and for teaching me all I wanted to know about viruses and their Universe. I also thank you for the extensive (voluntary) sampling of several different hypersaline environments around the world during your travels, so that we could only concentrate on the lab work.

I am grateful to Professor Kaarina Sivonen and Docent Sari Timonen for carefully reviewing my thesis and providing me with your excellent comments despite the tight schedule. I also thank Sari as well as Doctor Anne Ylinen for being in my Thesis Follow Up Group during the last three years. Thank you for your useful advices and all the good time we had over coffee, tea and sweets.

Viikki Graduate School in Molecular Biosciences is thanked for supporting my thesis project, for providing interesting and useful study courses and events, and for funding the travelling and participation to international conferences abroad.

I sincerely thank all my co-authors for such great projects we accomplished together. Docent Elina Roine and Professor Aharon Oren are thanked for the sampling of hypersaline environments in Israel. I am grateful to Aharon for sending me pictures of different hypersaline environments that I could use in the
thesis. I thank Doctor Maija Pietilä for your huge effort on the pleolipovirus work and for always having the latest knowledge of haloviruses. Thank you also for all the advices concerning the dissertation. I thank Professor Sarah Butcher as well as Doctor Violeta Manole and Lassi Liljeroos for your expertise on electron cryo-microscopy and tomography.

I wish to deeply thank our highly skilled technicians, Päivi Hannuksela, Sari Korhonen, Soile Storman, and Helin Veskiväli for your excellent technical support on this work. It has been a great joy to work with you as well as enjoy your delightful company during coffee breaks.

Docent Janne Ravantti is thanked for his valuable help with any kind of computerized analyses, as well as for the many occasions when computers or printers (or perhaps the user’s understanding) needed to be fixed. Thank you for always being ready to help during all those times you would have had much important work to do.

I thank all the past and present members on the Programme on Molecular Virology. Your help and skills are highly appreciated.

I thank my colleague and dear friend Ana Senčilo for many joyful moments we have had throughout these years. You have always been very supportive, encouraging and a good friend. I also thank Doctor Alesia Romanovskaya, Tatiana Demina and everybody else joining our wonderful, though very modest, “plastic box lunch company”. Although it is probably not advisable to laugh and eat at the same time, I always have such a great time with you guys.

I wish to thank my beloved friends, Anna, Sirke, Maria, Jemina, Susanna, and Mikko for bringing so much joy and delightful moments into my life.

My warmest appreciation goes to my dear family, my parents Tuula and Leo, my brother Toni and my sister-in-law Katri, my grandmother Kirsti, as well as our furry companions, Rocky and Henry. You are such an endless source of happiness, encouragement, support, and useful advices. I thank Toni especially for your help with the English language during thesis writing. I am very grateful to my late grandparents Evgenia and Professor Neno Atanassov for the inspiration to study biology.
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