DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS,
ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

27/2016

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Life in Extreme Environments: Physiological Changes in Host Cells During the Infection of Halophilic Archaeal Viruses



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DOCTORAL PROGRAMME IN MICROBIOLOGY AND BIOTECHNOLOGY
UNIVERSITY OF HELSINKI

Life in extreme environments: Physiological changes in host cells during the infection of halophilic archaeal viruses

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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 lecture room 106 (ls B5), Latokartanonkaari 7 (B-building), Helsinki, on the 9th of December 2016, at 12 o'clock noon.

Helsinki 2016

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Cover picture: Photography of Julija Svirskaitė

ISBN 978-951-51-2710-5 (paperback)
ISBN 978-951-51-2711-2 (PDF)
ISSN 2342-5423 (Print)
ISSN 2342-5431 (Online)

Hansaprint
Helsinki 2016

Behind every success

is effort...

Behind every effort

is passion...

Behind every passion is someone

with the courage

to try.

ORIGINAL PUBLICATIONS

List of the articles included in the thesis:

I. Svirskaitė J, Oksanen HM, Daugelavičius R, Bamford DH. 2016. **Monitoring physiological changes in haloarchaeal cell during virus release.** Viruses 8:59.

II. Demina TA, Pietilä MK, Svirskaitė J, Ravantti JJ, Atanasova NS, Bamford DH, Oksanen HM. 2016. **Archaeal *Haloarcula californiae* icosahedral virus 1 highlights conserved elements in icosahedral membrane-containing DNA viruses from extreme environments.** mBio 7(4):e00699-16.

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

The doctoral candidate's contribution to the articles included in this thesis:

I. JS conceived and designed the experiments, performed experiments, analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

II. JS contributed to the design and performance of virus life cycle experiments, as well as acquisition of data and its handling. JS also participated in drafting the manuscript and its revision.

ABBREVIATIONS

MCP – major capsid protein
ICTV – International Committee on Taxonomy of Viruses
PCB – phenyldicarbaundecaborane
NCBI – National Center for Biotechnology Information
VLP – virus-like particle
ATP – adenosine triphosphate
MGM – modified growth medium
DNA – deoxyribonucleic acid
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
ds – double-stranded
ss – single-stranded
TTSV1 – *Thermoprotheus tenax* spherical virus 1
PSV – *Pyrobaculum* spherical virus
ATV – *Acidianus* two-tailed virus
HCTV-1, 2, 5 – *Haloarcula californiae* head-tail viruses 1, 2, 5
HGTV-1 – *Halogranum* head-tail virus 1
HHTV-1, 2 – *Haloarcula hispanica* head-tail viruses 1, 2
HRTV-4, 5, 7, 8 – *Halorubrum* head-tail viruses 4, 5, 7, 8
HVTV-1 – *Haloarcula vallismortis* head-tail virus 1
HSTV-1 – *Haloarcula sinaiiensis* head-tail virus 1
HSTV-2 – *Halorubrum sodomense* head-tail virus 2
HCIV-1 – *Haloarcula californiae* icosahedral virus 1
HHIV-2 – *Haloarcula hispanica* icosahedral virus 2
STIV – *Sulfolobus* turreted icosahedral virus
STIV2 – *Sulfolobus* turreted icosahedral virus 2
HAPV-2 – *Haloarcula* pleomorphic virus 2
HHPV-1 – *Haloarcula hispanica* pleomorphic virus 1
HGPV-1 – *Halogeometricum* pleomorphic virus 1
HRPV-1, 2, 3, 6, 7, 8 – *Halorubrum* pleomorphic viruses 1, 2, 3, 6, 7, 8

SUMMARY

Archaea were discovered only 4-5 decades ago. The majority of archaea and their viruses originate from extreme environments many of which are characterized by thriving in extreme salinities. The number of isolated archaeal viruses is just a small fraction of the known viruses. Such a lack of knowledge warrants further studies on archaeal viruses and their life cycles. The exit of mature progeny viruses from the archaeal cell is the focus of this study. The viruses used in this study represent all known haloarchaeal virus morphotypes: icosahedral-tailed (HHTV-1), icosahedral-tailless (SH1, HCIV-1), spindle-shaped (His1) and pleomorphic (His2). Established technologies exist for virion characterization and virus life cycle determination (by using cell culture turbidity and external virus concentration measurements). Factors associated with membrane integrity, the binding of lipophilic anion phenyldicarbaunundecaborane (PCB), oxygen consumption and adenosine triphosphate (ATP) levels were used to extend the traditional methods for the life cycle studies.

These technologies were then utilized in the life cycle studies of HCIV-1, a recently isolated haloarchaeal virus with 12 virion structural proteins and an inner membrane. The internal membrane vesicle encloses a linear double-stranded DNA (dsDNA) genome of 31,314 bp. The genome sequence and its organization express a high similarity to the genomes of archaeal viruses in the *Sphaerolipoviridae* family. A rapid cell culture turbidity drop and increase of virus concentration in the cell culture medium took place when SH1, HHTV-1 and HCIV-1 exited the cell. The data also demonstrated the simultaneous binding of lipophilic PCB anions to cell debris, a lethal decrease in respiration and ATP leakage. All the measured properties support the conclusion that these three viruses have a lytic life cycle. However, His1 and His2 virus release did not significantly affect cell physiology suggesting that these haloarchaeal viruses cross the plasma membrane without depolarizing the cell. These results provide insights into the enigmatic and unique release mechanisms of haloarchaeal viruses and highlight the step forward in our understanding of archaeal viruses and their interactions with their host cell.

TABLE OF CONTENTS

ORIGINAL PUBLICATIONS ..... **I**

ABBREVIATIONS ..... **II**

SUMMARY ..... **III**

TABLE OF CONTENTS ..... **IV**

1. INTRODUCTION **1**

1.1 ORIGIN AND EVOLUTION OF THE THREE DOMAINS OF LIFE 1
1.2 ARCHAEAL TAXONOMY 2
1.3 VIRUSES 6
1.4 TAXONOMY OF VIRUSES 7
1.5 ARCHAEAL VIRUSES 8
1.5.1 Crenarchaeal viruses *8*
1.5.2 Euryarchaeal viruses *10*
1.6 MEASUREMENTS OF VIRAL GROWTH IN LIQUID 14
1.6.1 Measuring virus-induced cellular damage using lipophilic anions *14*
1.6.2 Measurements of dissolved oxygen and ATP *15*

2. AIMS OF THE STUDY **17**

3. MATERIALS AND METHODS **18**

4. RESULTS AND DISCUSSION **19**

4.1 VIRAL LIFE CYCLES 19
4.2 CHANGES IN CELL PHYSIOLOGY DURING VIRUS EXIT 20
4.3 DESCRIPTION OF HCIV-1 VIRUS 21

5. CONCLUSIONS AND FUTURE PERSPECTIVES **23**

6. ACKNOWLEDGEMENTS **24**

7. REFERENCES **26**

1. INTRODUCTION

1.1 Origin and evolution of the three domains of life

The tree of life is a simplified representation of evolutionary pathways. It contains three main branches represented by the three domains of life: *Eukarya*, *Bacteria* and *Archaea*. The *Archaea* branch is the latest addition to the tree of life (Woese and Fox, 1977; Woese et al., 1990). By researching thermophilic bacteria that are situated close to the root of the tree of life, it was found that they harvest their energy from hydrogen similarly to some archaea (Pace, 1991). It suggests that possibly the last common ancestor of these two domains also thrived at high temperatures and used hydrogen as an energy source (Weiss et al., 2016). Over time, the environment changed and abundance of ancestors adapted to varying habitats, which promoted deviation into different species (Lande, 2009; Wachtershauser, 2006). In such a way, the three domains formed with their own similarities and differences. Archaea are often extremophiles and they can occupy areas which are often less inhabited by bacteria and eukaryotes (Madigan and Oren, 1999). Such habitats include high acidic or alkaline lakes and springs in a wide range of temperatures. There are also mesophilic archaea, especially in oceanic ecosystems, but in comparison, the *Archaea* domain is still not as well explored as the *Bacteria* and *Eukarya* (DeLong, 1998).

Even though archaea look like bacteria, with a single cell morphology and an absence of nuclei, they are very distant in the evolutionary sense. They are also quite distant from the eukaryotes. However, archaeal RNA polymerases and transcriptional machineries are more comparable to the eukaryotic than to the bacterial ones (Klenk et al., 1992; Puhler et al., 1989; Woese et al., 1990).

Bacteria and eukaryotes build their membranes from lipids constructed out of fatty acid chains linked by ester bonds (Kaiser et al., 2011). Only the polar part of lipids remains conserved in cells belonging to all three domains. In order to survive in extreme environments archaeal cells have developed different ways to protect themselves (Dong and Chen, 2012; Gilmour, 1990; Gunbin et al., 2009; Oger, 2015; Sharma et al., 2007). One of the ways is to strengthen their envelope and lipid membrane. To reach such an evolutionary goal a more durable lipid complex from isoprenoids linked by ether junction was obtained (Derosa et al., 1986; Kates, 1992). The most abundant lipid in the membranes of archaeal methanogens and halophiles is diphytanyl glycerol diether, also named as archaeol (Albers

and Meyer, 2011; Derosa et al., 1986). There are also other types of lipids like caldarchaeol and dibiphytanyl glycerol tetraether mostly used by some methanogens and hyperthermophiles (Koga and Morii, 2007).

1.2 Archaeal taxonomy

Even though *Archaea* domain is still relatively newly discovered in comparison with *Bacteria* and *Eukarya,* already several inferred evolutionary relationships can be visualized in the branching phylogenetic tree of the archaea (Fig. 1). However, the origin of archaea is still debated (Petitjean et al., 2015a; Petitjean et al., 2015b; Raymann et al., 2015). Here I describe the current known phyla of archaea: *Thaumarchaeota, Crenarhaeota, Nanoarchaeota, Korarchaeota* and *Euryarchaeota* (Fig. 1).

It was thought in the late 1970s that *Archaea* consisted only of extremophiles belonging to *Euryarchaeota* adapted to grow in different extreme environments and the hyperthermophiles of the *Crenarchaeota* thriving in temperatures reaching 113 °C (Blochl et al., 1997; Kurr et al., 1991; Stetter, 1982). Numerous deeply branching archaea are hard to cultivate in controlled conditions, but culture-independent methods such as sequencing of the 16S rRNA genes have increased our knowledge. Only several years later Delong (1992) and Fuhrman et al. (1992), by analyzing 16S rRNA sequences, taken from mesophilic and psychrophilic environments, suggested that crenarchaea are much more diverse than previously thought. Further investigation led to the discovery of an unexpected symbiosis of *Cenarchaeum symbiosum* with marine sponges (Preston et al., 1996). Nonetheless, DeLong (1998) sequenced cell samples from different mesophilic environments and grouped them into, what at that time was called, "Group 1 *Crenarchaeota*". After the phylogenetic analyses of Brochier-Armanet et al. (2008), it became clear that a big group of "mesophilic *Crenarchaeota*" actually is a distinct separate phylum that is branching deeper than *Crenarchaeota*.

The diversity of the newly discovered thaumarchaea is extensive. Interestingly, thaumarchaeal isolates have been described to be capable of oxidizing ammonia (Konneke et al., 2005), while it was thought for a long time that only some proteobacteria have this capability. Another 'wonder' are filamentous multicellular thaumarchaea found in mangrove tropical swamps. This suggests an accomplished ectosymbiosis with bacteria (Muller et al.,

2010). It was suggested that the ancestor of *Thaumarchaeota* was thermophilic and because of horizontal gene transfer, adapted to a mesophilic lifestyle (Lopez-Garcia et al., 2004). At this moment the phylogeny of archaea is constantly shifting (Madigan et al., 2014). However, there are still some articles that describe crenarchaea that have mesophilic or psychrophilic-like lifestyle: for example, 'Octopus Spring nitrifying crenarchaeote OS70' (Tomova et al., 2011), 'crenarchaeote symbiont of *Axinella damicornis*' (Taylor et al., 2007). It appears that not all mesophilic crenarchaea have been shifted to *Thaumarchaeota* branch.

While exploring extreme habitats by using 16S rRNA sequence comparison for samples from Obsidian pool in Yellowstone National Park by Barns et al. (1996) a new Korarchaeal

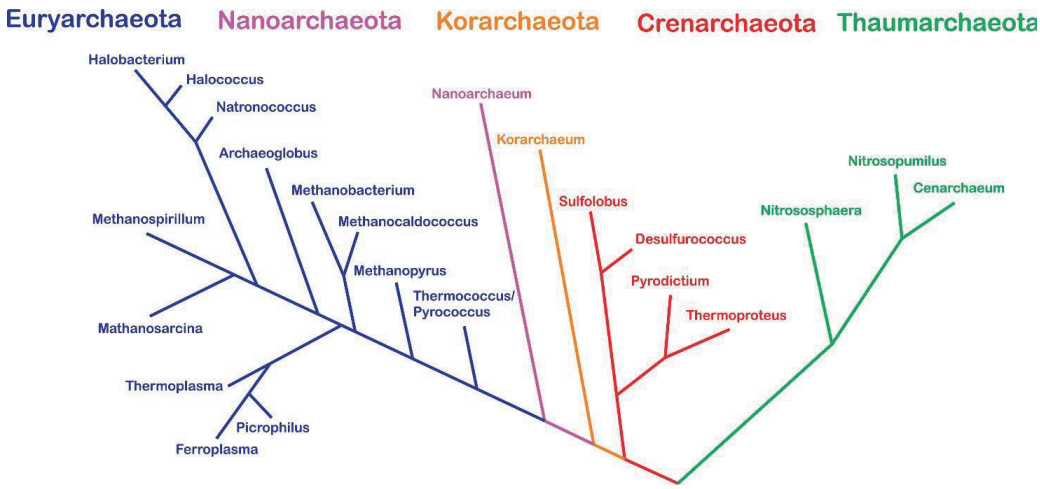


Figure 1. Tree of *Archaea* domain. Adapted from (Madigan et al., 2014).

branch was discovered. In addition, Miller-Coleman et al. (2012) probed the Great Basin Hot springs to determine the diversity and biogeography of korarchaea and summarized that most of the cells were found thriving in temperatures between 40 and 90°C. Suitable pH values varied from 5 to 8. Metagenomic screening made by Elkins et al. (2008) gave hints about the scavenger-like metabolism utilized by korarchaea. This analysis gave sufficient insight into the physiology of *Korarchaeum cryptofilum* to allow this organism to be introduced into culture in order to make electron micrographs to disclose that this cell is filamentous and prefers to grow in mat-like formations (Elkins et al. (2008).

Fricke et al. (1989) gathered rocks and gravel from the Mid-Atlantic ridge and, by using enrichment cultures, isolated a novel representative of crenarchaea phylum, *Ignicoccus* sp. strain KIN4/I. The surprise was that after examination under phase contrast light microscope they found that *Ignicoccus* had been attached by a smaller cocci, which later appeared to be a member of new archaeal phylum, *Nanoarchaeota* (Huber et al., 2002). The new cocci, *Nanoarchaeum equitans*, is only 0.35-0.5 μm long and has a 0.5 megabase genome. It was observed to be mostly attached in pairs to the *Ignicoccus* outer membrane. *N. equitans* possesses an S-layer and thrives in similar conditions as its host, 90 °C anaerobic environment enriched by S, H_2 and CO_2. It is suggested that these two members of archaea utilize a symbiotic lifestyle due to the observation that *N. equitans* does not grow without *Ignicoccus* and the *Ignicoccus* reproduction does not decline in the presence of *N. equitans* (Huber et al., 2003). It suggests that such a small genome cannot contain all genes needed for all the proteins required for independent survival.

Representatives of the *Euryarchaeota* branch share such qualities as thermophily (Cavalier-Smith, 2002; Darland et al., 1970), production of methane (Ferry et al., 1974; Zhou et al., 2014) and halophily (Gupta et al., 2015; Maturrano et al., 2006). Members of *Euryarchaeota* can be found even in places like the gut of thermite (Donovan et al., 2004) or even in the human mouth and gut (Horz and Conrads, 2010). Cultivation is still an important step to increase scientific knowledge about physiological specialization of an organism. Halophilic archaea are the easiest archaea to be cultivated in controlled environments when compared to thermophiles or methanogens. There are several archaeal model organisms representing genera as *Halobacterium, Haloferax,* and *Haloarcula* (Ma et al., 2010).

1.2.1.a) Halophilic archaea

Halophiles thrive in habitats where salt concentrations range from marine water (~3 % of salt) to nearly saturated salt solutions (~30 % of salt) (Fendrihan et al., 2006; Purdy et al., 2004). Such environments can be found for example in natural salt lakes, marine deep-water brines and salt fields. These ecosystems usually lack diversity, but are often rich in inhabitants. The uncomplicated cultivation of some halophiles has provided insights into the mechanisms that allow these microbes to withstand such stressful habitats. The cell

envelope plays a central role in the survival of archaea. The archaeal envelope, surface layer together with a more durable membrane (see above 1.1), successfully secures the cell from many stressful factors. The so-called S-layer can be present in bacteria as well, but it is particularly common in archaea (Klingl, 2014). The S-layer is made mostly out of proteins with different heterosaccharide chains and it forms two-dimensional crystals. It is arranged in units that are composed of two to six smaller identical components organized in different symmetries (Sleytr et al., 1999). Such formations can contain various additives like glycosides that can strengthen the envelope structure. Haloarchaea were the first archaea to be used in research on the prokaryotic S-layer (Houwink, 1956; Mescher and Strominger, 1976; Sumper et al., 1990).

The S-layer is firmly anchored to the plasma membrane having a pseudo transmembrane space in between (Albers and Meyer, 2011). The S-layer and the cell membrane have important roles to keep cells alive in such osmotic extremities. The plasma membrane harbors also different proteins that help to keep haloarchaea osmotic pressure in balance. Usually haloarchaea have a pinkish color because of pigmented bacteriorhodopsin and/or halorhodopsin. Sunlight powered bacteriorhodopsin takes part in pumping hydrogen protons out of the cell while halorhodopsin is needed to import chloride ions inside the cell (Schobert and Lanyi, 1982). This created hydrogen gradient is used to carry sodium ions across the membrane out in exchange into hydrogen ion back. The chain goes on and the sodium gradient allows the intracellular potassium to balance osmotic pressure (Fig. 2). This allows haloarchaea to transform light energy into chemical energy in a process unrelated to photosynthesis. Adenosine triphosphate (ATP) is produced during this process (Gilmour, 1990). Such a unique way of survival, distinct from bacteria, allows these cells to thrive where no one previously expected to encounter life. Archaea are infected by a number of viruses that are adapted to their hosts' environment.

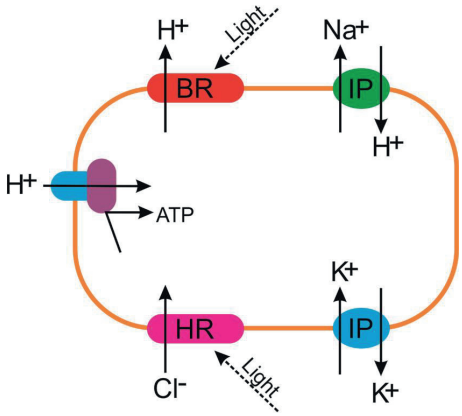


Figure 2. Archaeal mechanisms involved in high salinity survival. Adapted from (Gilmour, 1990). BR, bacteriorhodopsin; HR, halorhodopsin; IP, ion pumps; and ATPase.

1.3 Viruses

Viral research is traditionally heavily biased towards pathogenic viruses infecting humans, livestock, and economically important plants. Presently virology is blooming because of improved methods like electron microscopy, polymerase chain reaction, next generation sequencing, and bioinformatics.

What is a virus? The traditional way to describe a virus is that it is a nonliving particle carrying the viral genome, which is shielded most often by a protein capsid and can replicate using the host's machinery. Virion is a mature infective particle residing outside of its host. The more we discover about viruses the more boundaries and rules are bent. For example, not all viruses are encapsulated by a protein capsid, and not all viruses use the same replication and transcription machineries (Baltimore, 1971; Pietilä et al., 2012). Some viruses bring their own enzymes to the host cell. Furthermore, not all viruses are small and some viruses can even be infected by virophages (Aherfi et al., 2016; Webster and Granoff, 1997). However, all viruses are obligatory parasites.

The most important parts of a virus are the genome and the capsid. The viral genome can consist of DNA or RNA, which can be double-stranded (ds) or single-stranded (ss). Viruses with RNA genomes are the fastest to adapt to environmental changes, but DNA viruses are genetically more stable (Elena and Sanjuan, 2005; Shadan and Villarreal, 1995; Steinhauer and Holland, 1987). The genome of the virus is its essence and all other viral tools only help to protect and replicate it. Viruses generally share the same habitat as the host, so if the host is an extremophile then the virus has to adapt to live in the same conditions. In some cases, there are internal or external membranes in the virus particles, which not only help to protect the genome, but are also important during virus entry and exit (Delima et al., 1995; Garoff et al., 1998; Sun and Wirtz, 2006).

Viral multiplication cycle can be broken down into several steps. First, the virus must gain entry to the host cell usually through recognition of a receptor on the host cell surface and irreversibly bind to the host cell followed by an entry to the cell (Yin et al., 2010). Different viruses have specific host ranges (Novikov and Atabekov, 1970). The entry process of the viral genome into the host cell depends on virus morphology and host cell properties. The speed of the adsorption and entry can differ depending on many factors, including environmental ones (Kukkaro and Bamford, 2009). As an example it was noticed that the

adsorption rate of halophilic viruses is 10 or even 100 times slower than some freshwater phages (Kukkaro and Bamford, 2009). This can be attributed to the high viscosity of brines. Surprisingly, some viruses from acidic hot springs can adsorb in seconds (Quemin et al., 2013). It is possible, that in acidic hot springs, viruses had to adapt to fast adsorption to spend less time in the harmful environment.

After a successful entry, the replication, transcription and translation take place. The late produced structural proteins in some cases will self-assemble to precursor particles that are capable for packaging the genome (Rong et al., 2011). Newly made mature virions can exit the cell in many different ways which can leave the cell dead or alive (Komarova, 2007). In addition, within both subcategories there are other variables in virus exit such as speed and synchrony. Some viruses exit slowly and in non-synchronized manner making it difficult to define the type of exit. As an example some thermophilic viruses have been shown to create pyramid-like structures incorporated into the host plasma membrane to help with the exit of virus particles (Prangishvili, 2013). There are many variations in the life cycles of different viruses that operate successfully in their specific hosts. As viruses are not considered to be alive in the conventional sense, the fact that they evolve makes them eligible for their own taxonomic system.

1.4 Taxonomy of viruses

The way living organisms are classified taxonomically has changed over time due to the more precise evolutionary reconstructions made possible by the 16S rRNA sequencing. Viruses do not possess ribosomes due to their relatively small genome size, it is difficult to unravel their evolutionary origins by sequence comparison. However, viruses evolve rapidly and there is much debate about which is the most convenient way to classify them. The Baltimore classification system is based on the viral genome type and its replication mechanism (Baltimore, 1971). A competing system, where viruses are placed into species, genera, families, and orders based on many aspects (presence of an envelope, virion morphology, host genome sequence and etc.), is provided by the International Committee on Taxonomy of Viruses (ICTV). The newest idea regarding virus grouping is based on the observation that there is a limited number of virion architectures (Abrescia et al., 2012; Bamford et al., 2002). New virus isolates bring new ideas on how to organize the virosphere.

Keeping in mind that viruses exceed in numbers all the cells there are always viruses trying to infect appropriate hosts (Bergh et al., 1989). The newest additions to the tree of life are archaea, and their viruses often have peculiar morphotypes.

1.5 Archaeal viruses

The first archaeal virus was isolated in 1970's before the recognition of the *Archaea* as a domain of life (Torsvik and Dundas, 1974). However, these viruses did not raise particular interest because they bore similarities to the most common icosahedral-tailed bacteriophages. For a long time after the accidental discovery of archaeal viruses, there was very little activity on the field. However, recently the number of archaeal viruses was significantly increased (Atanasova et al., 2015a; Atanasova et al., 2012). The most successfully isolated and studied archaeal viruses infect cells belonging to the two biggest branches of the *Archaea* (*Crenarchaea* and *Euryarchaea*) (Prangishvili, 2013; Prangishvili et al., 2006a).

1.5.1 Crenarchaeal viruses

Crenarchaeal viruses sparked interest due to the exceptional capsid structures and other features that were not found in any other viruses (Pina et al., 2011; Prangishvili et al., 2016). Crenarchaeal viruses can be at least linear, spindle-shaped, droplet-shaped, spherical, icosahedral, and bottle-shaped. The numerous distinct morphologies of crenarchaeal viruses are not characteristic for either bacterial or eukaryotic viruses (Pietilä et al., 2014; Prangishvili, 2013). The only morphologies that are common for bacterial, eukaryotic and archaeal viruses are filamentous and icosahedral ones (Atanasova et al., 2015b; Nasir et al., 2014). Often virus-like particles (VLP) in environmental samples are morphologically much more diverse than the actual viruses isolated from these samples (Sime-Ngando et al., 2011). Even though icosahedral-tailed viruses have been visualized in samples derived from hyperthermophilic environments, they have been successfully isolated only for euryarchaeal host cells (Fig. 3) (Pina et al., 2011; Rachel et al., 2002). Icosahedral-tailless viruses have been described for both, crenarchaeal and euryarchaeal cells

(Happonen et al., 2010; Porter et al., 2005). The family *Turriviridae* contains *Sulfolobus* turreted icosahedral virus (STIV) which thrives in hyperthermophilic habitats (Fu and Johnson, 2012; Prangishvili, 2013). An interesting feature of these viruses is an inner membrane which is surrounded by an icosahedral capsid ornamented by spikes reminiscent to enterobacterial phages like PRD1 (Bamford and Bamford, 1990; Veesler et al., 2013). Another interesting virus morphotype infecting hyperthermophilic archaeal cells is a simple sphere. The linear genome is packed into a helical nucleocapsid and the virion is enclosed by a lipid envelope. Such spherical archaeal viruses, like the described *Thermoprotheus tenax* spherical virus 1 (TTSV1) and *Pyrobaculum* spherical virus (PSV), belong to the *Globuloviridae* family (Häring et al., 2004).

The observed abundance of linear VLP's in the electron micrographs taken from environmental samples is incomparable with the low number of isolated linear viruses. Linear VLPs were noticed in hyperthermophilic and in hypersaline environments, but so far

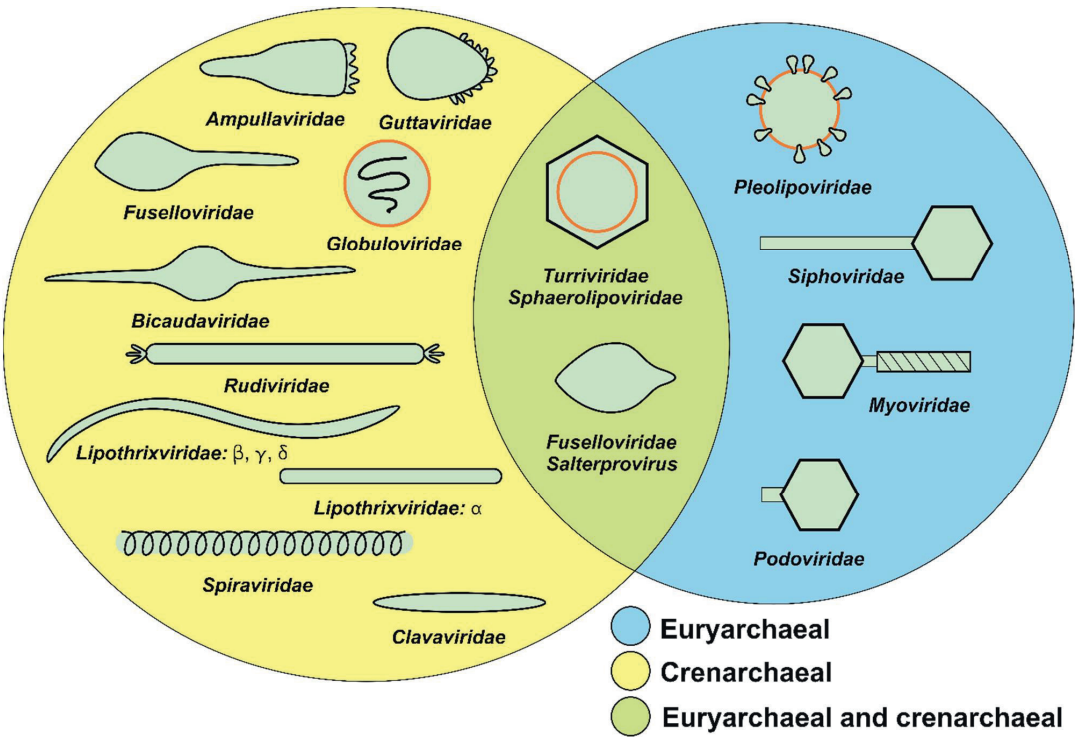


Figure 3. Currently known crenarchaeal and euryarchaeal virus morphologies and the virus families.

only such viruses infecting the hyperthermophilic creanarchaea have been successfully isolated (Fig. 3) (Bettstetter et al., 2003; Prangishvili et al., 1999; Sime-Ngando et al., 2011). The order of linear dsDNA viruses, *Ligamenvirales*, contains two families: *Lipothrixviridae* of flexible viruses and *Rudiviridae* of rigid ones (Häring et al., 2005b; Prangishvili et al., 1999; Prangishvili and Krupovič, 2012). There are at least two more representatives of linear viruses: those with bacilliform (*Clavaviridae*) or coil-shaped (*Spiraviridae*) morphologies (Fig. 3) (Mochizuki et al., 2012; Mochizuki et al., 2010).

Unlike tailed and linear viruses, the spindle-shaped viruses have been isolated from both thermophilic and halophilic environments (Fig. 2) (Bath et al., 2006; Krupovič et al., 2014; Redder et al., 2009; Xiang et al., 2005). Spindle-shaped viruses can have one short or long tail, or even two long tails at the opposite extremities. Hyperthermophilic spindle-shaped viruses with a short or long tail infecting crenarchaeal cells are positioned into the *Fuselloviridae* family (Bath and Dyall-Smith, 1998). The only halophilic short-tailed spindle-shaped virus, His1, belongs to the *Salterprovirus* genus (Bath et al., 2006). However, based on recent taxonomic analyses of viral structural proteins it has been proposed that His1 could be placed into the *Fuselloviridae* family (Krupovič et al., 2014). The two-tailed viruses have their own family named *Bicaudaviridae* and the only member is *Acidianus* two-tailed virus (ATV)**.** This is the only known virus that undergoes extracellular changes (Prangishvili et al., 2006c). Another strain from *Acidianus* genus is also infected by another virus with an extraordinary bottle-shaped morphology that belongs to the *Ampullaviridae* family (Häring et al., 2005a). In addition, *Sulfolobus* infecting droplet-shaped virus was isolated from solfataric fields and was immediately proposed to have its own designated family *Guttaviridae* (Fig. 3) (Arnold et al., 2000).

1.5.2 Euryarchaeal viruses

Euryarchaeal viruses are morphologically not as diverse as the crenarchaeal ones, but they have brought new insights regarding evolution, genetic variety, morphology, and survival (Prangishvili et al., 2016). All the earliest isolated and cultivated euryarchaeal viruses have an icosahedral-tailed morphology. It was expected that this was the most common or even the only displayed euryarchaeal virus morphology (Schnabel et al., 1982; Torsvik and Dundas, 1974; Witte et al., 1997). Nevertheless, with further research viruses

with icosahedral-tailed, icosahedral-tailless, spindle-shaped, and pleomorphic morphologies were isolated (Dellas et al., 2014; Roine and Oksanen, 2011).

1.5.2.a) Icosahedral-tailed euryarchaeal viruses

Icosahedral-tailed viruses are the most commonly isolated viruses amongst euryarchaea and account for more than half of the known virus isolates (Atanasova et al., 2015a; Atanasova et al., 2012). Included in this group are three subtypes of icosahedral-tailed viruses: myoviruses with a long contractile tail (*Myoviridae*), siphoviruses with a long non-contractile tail (*Siphoviridae*), and podoviruses with a short non-contractile tail (*Podoviridae*). Amongst archaeal icosahedral-tailed viruses the most abundant are myoviruses, which account for about 80% of all known archaeal icosahedral-tailed viruses (Atanasova et al., 2015a; Dellas et al., 2014) . However, archaeal icosahedral-tailed viruses rarely dominate in environmental samples (Sime-Ngando et al., 2011). The most studied icosahedral-tailed viruses are ΦH (*Halobacterium salinarum*), ΦCh1 (*Natrialba magadii*), HF1 (*Haloferax volcanii*) and HF2 (*Halorubrum coriense*) (Klein et al., 2002; Nuttall and Dyall-Smith, 1993; Stolt and Zillig, 1994). Nevertheless, new viruses emerge regularly and will be described in due course. The following archaeal icosahedral-tailed viruses, HCTV-1, HCTV-2, HCTV-5, HGTV-1, HHTV-1, HHTV-2, HRTV-4, HRTV-5, HRTV-7, and HRTV-8 have their genomes sequenced and analyzed by Senčilo et al. (2013). Three more tailed viruses HVTV-1, HSTV-2, and HSTV-1 have been studied in more detail. Their life cycles, genome sequences, and virion architectures were determined (Pietilä et al., 2013b; Pietilä et al., 2013c). All these viruses have a dsDNA genome that is either circularly permuted or contain direct terminal repeats. During these studies it has been shown that all known archaeal icosahedral-tailed viruses (similarly to bacterial ones) lyse cells during progeny egress (Pietilä et al., 2013b; Pietilä et al., 2013c; Tang et al., 2004; Witte et al., 1997). It is noteworthy that even though some viruses were isolated from very distant geographical areas, their genomic profiles were astonishingly similar (Senčilo et al., 2013). Even though databases are scarce for archaeal virus genome sequences, some proteins have been successfully identified due to the structural similarity between bacterial and archaeal icosahedral-tailed virus proteins (Krupovič et al., 2010; Senčilo et al., 2013).

1.5.2.b) Icosahedral tailless euryarchaeal viruses

At present five icosahedral-tailless haloarchaeal viruses having an inner membrane have been isolated (SH1, PH1, HHIV-2, HCIV-1 and SNJ1), SH1 being the first isolated model virus in this group (Atanasova et al., 2015a; Jaakkola et al., 2012; Porter et al., 2005; Porter et al., 2013; Zhang et al., 2012). These virion morphotypes share a very similar structure with the crenarchaeal icosahedral-tailless viruses STIV and STIV2 (Fig. 3) (see also 1.4.1). SH1, PH1 and HHIV-2 infect the same *Haloarcula hispanica* strain while SNJ1 replicates in several *Natrinema* strains and HCIV-1 in *Haloarcula californiae*. Initially it was thought that SNJ1 is an icosahedral-tailed virus (Mei et al., 2007). However, later it was confirmed that SNJ1 belongs to the icosahedral-tailless viruses (Zhang et al., 2012) that all belong to the *Sphaerolipoviridae* family. The most recently isolated such virus, HCIV-1, most probably belongs to this family as well (Atanasova et al., 2015a) (see also results and discussion). These viruses have the same exit strategy (host cell lysis) (see also results and discussion). There are controversial data about the SH1 progeny exit strategies. It was first considered to be a lytic virus but later studies support the proposal that it is non-lytic (Porter et al., 2005; Porter et al., 2007). HHIV-2 is clearly lytic as cell culture turbidity and viable cell count drops and there is a quick increase of free virus particles in the medium (Jaakkola et al., 2012). PH1 virus is lytic, but the cell lysis is delayed (Porter et al., 2013). SNJ1 is characterized as lysogenic virus that replicates as a plasmid (Mei et al., 2007; Zhang et al., 2012).

1.5.2.c) Spindle-shaped euryarchaeal virus

Amongst haloarchaeal viruses, there is only one isolated spindle-shaped virus, His1. It was isolated from Australian saltern and infects *Har. hispanica* and belongs to the *Salterprovirus* genus (Bath and Dyall-Smith, 1998; Pina et al., 2011). It is unexpected that there is only one spindle-shaped virus isolated from hypersaline habitats as environmental samples are populated by VLPs having this morphology (Sime-Ngando et al., 2011). His1 virus capsids are constructed out of one major lipid-modified protein species and three minor ones (Bath and Dyall-Smith, 1998; Pietilä et al., 2013a). It has been shown that His1

progeny egress only retards host growth, but it is still unknown how this virus actually exits and what damage it does to the cells (Pietilä et al., 2013a).

1.5.2.d) Pleomorphic euryarchaeal viruses

Pleomorphic euryarchaeal viruses have a flexible morphology (greek πλέω- more, and -μορφή form). At present about 10 pleomorphic haloarchaeal viruses have been published: HRPV-1, 2, 3, 6, 7, 8 infecting *Halorubrum* strains, HAPV-2 infecting a *Haloarcula* strain, HHPV-1 and His2 infecting *Har. hispanica* and HGPV-1 infecting a *Halogeometricum* strain (Atanasova et al., 2015a; Atanasova et al., 2012; Bath et al., 2006; Pietilä et al., 2012; Pietilä et al., 2009; Prangishvili et al., 2006b). The first and best described virus is HRPV-1 (Pietilä et al., 2012; Pietilä et al., 2010; Pietilä et al., 2009). All of the pleomorphic viruses, except for HRPV-7, 8, and HAPV-2, have been well characterized in terms of life cycle, virion structure, composition, stability and host range, as well as genomic analysis (Atanasova et al., 2012; Pietilä et al., 2012; Roine et al., 2010; Senčilo et al., 2012). The other three viruses His2 virus was isolated in Victoria (Australia) and it was previously thought that it is a spindle-shaped virus together with His1 which was isolated from the same environment (Bath et al., 2006). All these viruses are made of different types of genomes (single-stranded circular or double-stranded linear or circular DNA) enveloped by a membrane vesicle. Vesicle is protein-rich having typically two protein species: small membrane protein and large spike protein (Pietilä et al., 2012). They probably acquire their lipids unselectively from their hosts during a budding process while exiting the host cell. It is expected that these viruses bud through the plasma membrane without host cell lysis.

1.6 Measurements of viral growth in liquid

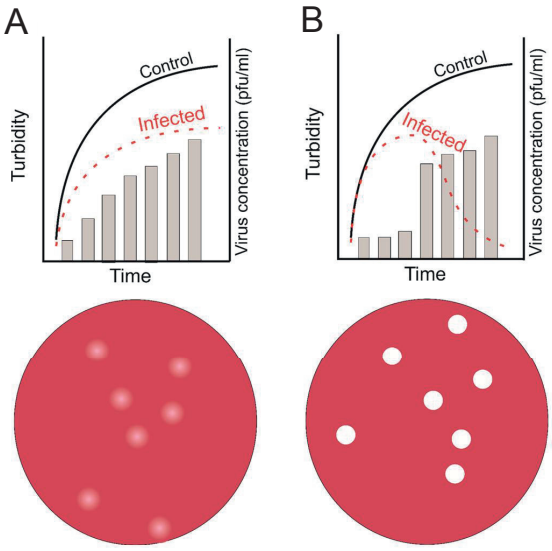


Figure 4. Virus growth measurements by using turbidity (top) and plaque assays (bottom). A – Non-lytic virus life cycle; B – Lytic virus life cycle. Columns indicate virus counts (pfu/ml).

Culture turbidity assays are commonly used to follow virus replication in infected cells (Fig. 4). The viruses that are released from cells without damaging the host typically form hazy plaques and the viral release is usually gradual (Fig. 4A). The turbidity of the infected culture in these cases decreases compared to the uninfected cultures. Another exit strategy is through cell lysis resulting in the rapid release of viruses. In such cases, plaques are more transparent or clear (Fig. 4B). There are also cases where empty host shells accumulate and turbidity measurements are harder to interpret (Bize et al., 2009; Brumfield et al., 2009).

1.6.1 Measuring virus-induced cellular damage using lipophilic anions

Phenyldicarbaundecaborane anion (PCB^-) is a lipophilic anion that irreversibly binds to exposed lipid bilayers and thus can be used as an indicator of cell integrity (Daugelavičius et al., 1997) (Fig. 5). PCB^- concentration can be determined using on-line electrochemical measurements in liquid cell cultures as utilized in some studies of virus life cycles (Jakutytė et al., 2012; Krupovič et al., 2007). This is achieved through the use electrodes and specially synthesized ion selective membranes sensitive to changes in PCB^- concentration. The concentration of PCB^- solution is higher (1 mM) in the glass electrode compared to the concentration in the medium (3 μM). The charge of the cell interior is negative due to ion pumps (Gilmour, 1990). Accordingly, the PCB^- cannot enter the intact cell (Fig. 5A). If cell damage occurs lipid bilayers become exposed leading to PCB^- binding and removal of free

PCB^- from the external medium (Fig. 5B, 6B). Completely destroyed cells (pre-lysed) with most of their envelopes disrupted immediately bind maximal amounts of PCB^-. When the intact cells are placed in fresh medium the PCB^- concentration initially drops due to natural

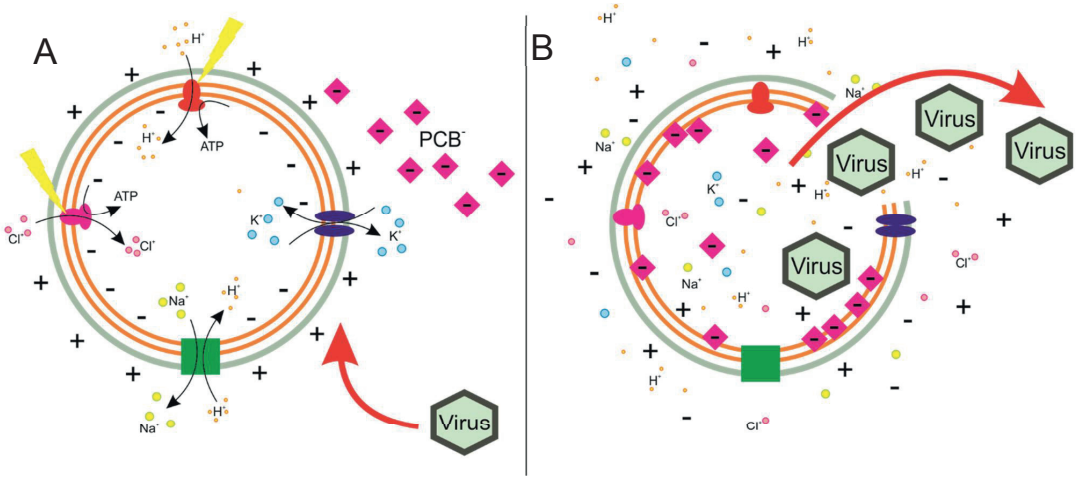


Figure 5. Schematic presentation of PCB anion binding to cell lipid bilayer and its connection to viral exit. A – Pre-viral cell state; B – Post-viral cell state.

culture aging leading to the formation of some cell debris (Fig. 6A control and infected). This process is depicted in Figure 6A.

1.6.2 Measurements of dissolved oxygen and ATP

The dissolved oxygen concentration in the culture medium can be measured real-time using commercially available oxygen sensitive electrodes (Krupovič et al., 2007). When there are no cells in the medium during aeration the oxygen level is considered 100%. When cells are added respiration starts and oxygen level decreases. Respiration declines when cells approach stationary phase (Fig. 6B Control (a)). Actively respiring cells might even exhaust all the available oxygen (~0 %) (Fig. 6B Control (b)). When virulent viruses cause cell lysis oxygen levels approach ~100% once more (Fig. 6B, Infected). Pre-lysed cells do not consume any oxygen (Fig. 6B, Pre-lysed). At the end of the experiment sodium bisulfite is added to obtain a calibration point when all oxygen has been exhausted.

In addition to the electrochemical experiments, ATP can also be measured when analyzing the virus-induced changes in the cells (Krupovič et al., 2007). ATP is a universal

energy carrier and resides in the cell interior. When a virus lyses the cell, ATP is released into the medium. Prior to the measurement the cells are lysed using manufacturer's instructions and after that mixed with luciferin-luciferase blend creating light emission when in contact with ATP, which can be detected by luminometric measurements.

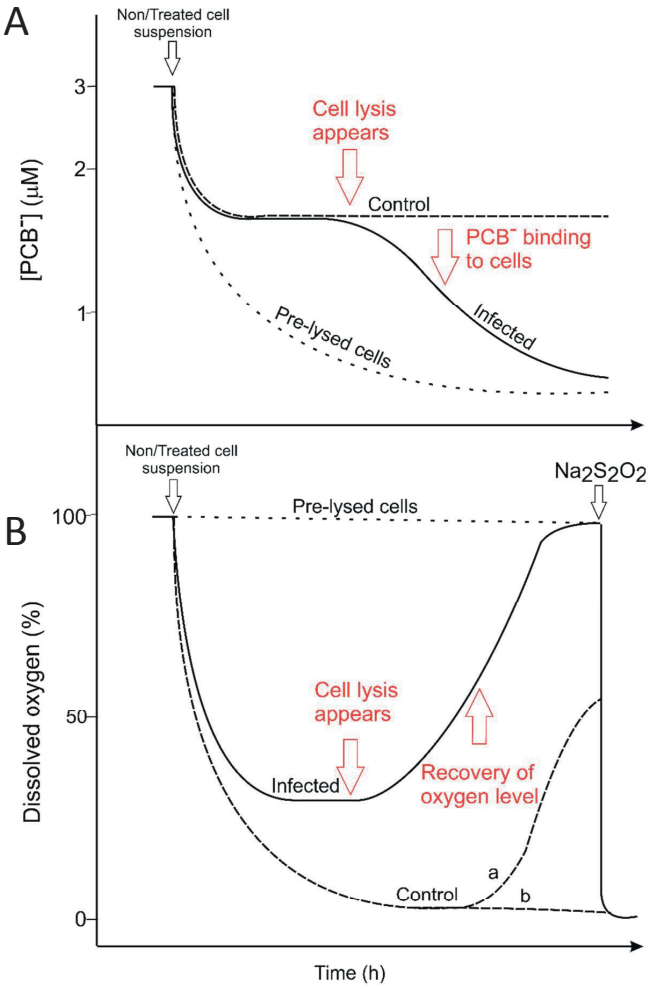


Figure 6. Interpretation of electrochemical measurements. A – PCB^-; B – Dissolved oxygen; a and b, see explanation in the main text.

2. AIMS OF THE STUDY

Archaea and their viruses have been discovered about 50 years ago. Due to this and the small research community working on them the accumulated knowledge is limited, compared to what is known about viruses that infect bacterial or eukaryotic cells. This was my reasoning to start working on viruses from extreme environments where novel virus types have been isolated. Studies of new viruses are of high academic interest for expanding the knowledge about the surrounding virosphere. More specifically, I concentrated on the archaeal virus exit mechanism which is an important part of the virus life cycle and interaction with its host. In the previous research, there were discrepancies about the exit mechanisms of archaeal viruses and my plan is to clarify such inconsistencies.

The specific aims of this study are summarized below:

- To adjust and use electrochemistry and ATP measurements to follow virus-host interactions in high salinity environments
- To observe physiological changes in haloarchaeal cells during infection with novel viruses
- To clarify different haloarchaeal virus exit mechanisms.

3. MATERIALS AND METHODS

Table 1. Archaeal viruses used in this study.

Virus	References
SH1	(Porter et al., 2005; Porter et al., 2007)
Haloarcula hispanica tailed virus 1 (HHTV-1)	(Kukkaro and Bamford, 2009; Senčilo et al., 2013)
Haloarcula hispanica virus 1 (His1)	(Bath and Dyall-Smith, 1998; Pietilä et al., 2013a)
Haloarcula hispanica virus 2 (His2)	(Bath et al., 2006; Pietilä et al., 2012)
Haloarcula californiae icosahedral virus 1 (HCIV-1)	(Atanasova et al., 2015a)

Table 2. Archaeal strains used in this study.

Name	Host for	References	Used in
Haloarcula hispanica	SH1, HHTV-1, His1, His2	(Juez et al., 1986)	I
Haloarcula californiae	HCIV-1	(Javor et al., 1982)	II

Table 3. Summary of methods used in this study.

Methods	Used in
Cultivation of halophilic prokaryotes and their viruses	I, II
Plaque assay	I, II
Virus adsorption assay	I, II
Virus life cycle analysis	I, II
Electrochemical and ATP measurements of cell cultures	I, II
Optical microscopy	II
Bradford assay for measuring protein concentration	II
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	II
Coomassie blue staining of proteins	II
Pro-Q Emerald and SYPRO-Ruby staining of proteins	II
Sudan Black B staining of lipids	II
Agarose gel electrophoresis and ethidium bromide staining	II
Extraction of lipids and thin-layer chromatography (TLC)	II
N-terminal sequencing of proteins	II
Mass spectrometry of proteins	II
Negative staining and transmission electron microscopy (TEM)	II
Thin-section TEM	II
DNA extraction and enzymatic digestion	II
Sanger sequencing and genome annotation	II
Purification of virus particles	II
Stability tests of the virion	II

4. RESULTS AND DISCUSSION

Hypersaline environments are rich with halophilic archaea (Oh et al., 2010). The most abundant halophilic archaea belong to the genera *Haloarcula*, *Halorubrum*, *Haloquadratum*, and *Haloferax* strains (Sime-Ngando et al., 2011). Viruses that infect halophilic archaea are also plentiful in these environments. However, only four viral morphotypes have been isolated from such environments: icosahedral-tailless, icosahedral-tailed, spindle-shaped and pleomorphic (Fig. 3) (Dellas et al., 2014). Several viruses with different morphologies can infect a single host strain (Atanasova et al., 2012). Five archaeal viruses were studied in this work: SH1, HHTV-1, His1 and His2 infecting *Haloarcula hispanica* (**I**) and HCIV-1 infecting *Haloarcula californiae* (**II**). These viruses represent all the currently known haloarchaeal virus morphotypes and utilize different approaches to leave the host cell when the progeny are mature. However, there are discrepancies in the literature about which exit methods some of these viruses use (Porter et al., 2005; Porter et al., 2007). In this work traditional (turbidity of the cell culture and plaque assay based measurements) and alternative (electrochemical on-line measurements and ATP assay) methods were adapted to follow the archaeal virus egress process in high salinity (**I**). Previously such on-line approaches have been successfully used on bacteria but so far not for cells requiring high salinity (Daugelavičius et al., 1997; Jakutytė et al., 2012).

4.1 Viral life cycles

During this study growth of *Haloarcula hispanica* and *Haloarcula californiae* strains (infected and non-infected) were monitored by applying culture turbidity measurements. In addition, the number of viable cells and progeny viruses were determined. Turbidity (A_{550}) of the non-infected cultures of *Har. hispanica* and *Har. californiae* reached ~1.4 (~2×10^9 cfu/ml) and ~1.3 (~1.5×10^9 cfu/ml), respectively (**I,** Figs. 2 A, D 3 A, D; **II,** Fig. 1 G). The viral one-step growth curve showed a turbidity drop in the cultures infected by SH1 (5 h post infection (p.i.)), HHTV-1 (7 h p.i.) and HCIV-1 (12 h p.i.) (**I,** Figs. 2 A, D 3 A, D; **II,** Fig. 1 G). The observed turbidity drop matched with the rate of progeny egress. Released viruses reached ~2.5×10^{11} pfu/ml (SH1), ~7.5×10^{10} pfu/ml (HHTV-1) and ~1×10^{10} pfu/ml (HCIV-1). Cultures infected by His1 and His2 did not show any turbidity drops while only slight

retardation of cell growth was observed (Bath et al., 2006; Pietilä et al., 2012; Pietilä et al., 2013a). Progeny release of both viruses began at ~4 h p.i. and reached ~3×10^{10} pfu/ml and ~5×10^{11} pfu/ml, respectively (**I,** Fig. 3 A, D). Among the currently known viruses, it appears that based on the turbidity measurements and plaque assays, there are two different haloarchaeal virus exit mechanisms: cell lysis and budding. However, more information is needed to examine this in more detail (**I**, see 3.2).

4.2 Changes in cell physiology during virus exit

Electrochemical experiments were carried out in the rich, highly saline MGM medium with added PCB^- using constant aeration at 37 °C. The presence of PCB^- in the medium did not disturb the production of viruses or cause significant changes in the host growth (data not shown). The timing of the PCB^- binding matched with the turbidity decrease for SH1, HHTV-1 and HCIV-1 viruses (**I,** Figs. 2 B, E; **II,** Fig.1 H) suggesting that the lipid bilayer was damaged and exposed allowing PCB^- to bind. However, in the case of His1 and His2 viruses there was no PCB^- binding even when progeny viruses were produced in high numbers (**I,** Figs. 3 B, E). This suggests that His1 and His2 viruses can cross the plasma membrane without disrupting the cellular lipid bilayer.

Non-infected *Har. hispanica* cells used less oxygen closer to the stationary phase while *Har. californiae* actively respired during entire growth in liquid culture (**I,** Figs. 2 C, F 3 C, F; **II,** Fig. 1 I). During infection with SH1, HHTV-1 and HCIV-1 oxygen levels in the medium increased together with PCB^- binding and turbidity drop (**I,** Fig. 2 C, F; **II,** Fig. 1 I). His1 and His2 did not have an effect on respiration (**I,** Fig. 3 C, F). This also suggests that His1 and His2 do not disturb the cell physiology during the virus release, whereas SH1, HHTV-1 and HCIV-1 cause a major collective collapse of respiration simultaneously with the turbidity decrease. Viruses are released and PCB anion binds to the exposed lipids. During SH1, HHTV-1 and HCIV-1 exit, it was observed that cell walls became permeable to ATP, whereas His1 and His2 egress did not show any measurable ATP release from the infected cells (**I,** Fig. 4 C; **II,** Fig. 1 F).

Here it was shown that the model virus SH1 causes considerable cellular damage during exit (**I**). Traditional measurements indicate major decrease in the cell culture turbidity matching with the release of progeny viruses. At the same time, lipophilic PCB anion binds

to the exposed lipid bilayer also pointing towards a lethal damage to the cell envelope. A decrease in oxygen consumption indicated a failure in the respiratory process, which also coincided with viral release. ATP leakage from the cells also coincided with progeny release indicating that SH1 is a virulent virus, as all the parameters used here support this conclusion (**I**) (Porter et al., 2005; Roine and Oksanen, 2011). The previous literature is not coherent in this matter as also a non-lytic behavior has been proposed (Porter et al., 2005; Porter et al., 2007). Viruses HHTV-1 and HCIV-1 utilize an analogous lytic life cycle than SH1, as indicated by similar behavior in all the used measurements (**I,** Figs. 2 A-F; **II,** Figs. 1 F-I).

Surprisingly, His1 and His2 viruses have a completely different exit mechanism compared to the SH1-type of viruses. The turbidity of the infected cultures does not considerably decrease in association with the virus release (Bath et al., 2006; Bath and Dyall-Smith, 1998; Pietilä et al., 2012). With His1 and His2 there were no PCB^- binding, changes in the oxygen consumption or release of ATP during the life cycle (**I**). It indicates that His1 and His2 cross the cell wall without damaging it, although the number of released viruses in both cases is high. One of the hypothesis on how these viruses exit the cell is budding. The multi-measurement approach used here enables the specific detection of the physiological events that occur during viral infection and it can be successfully adapted to other virus-host systems as well. The availability and practicality of this instrumentation enabled the detection of a viral exit mechanism that did not affect cell integrity. The usage of a number of complementing methods has opened up a new research avenue for studying the viral exit mechanisms used to cross the archaeal cell membrane.

4.3 Description of HCIV-1 virus

Archaeal icosahedral-tailless viruses are poorly characterized and require more studies to reveal the basic characters of such viruses. One such new virus is HCIV-1 that was acquired from a solar saltern in Thailand (Atanasova et al., 2015a). It was isolated with *Har. californiae* cells, but it also infects three additional euryarchaeal strains out of the 44 tested ones, thus demonstrating a fairly narrow host range. The HCIV-1 adsorption is relatively slow (~80% of particles adsorbed in ~5 hours), as with many other haloarchaeal viruses (**II,** Fig. S2 A) (Kukkaro and Bamford, 2009). In the early stages of infection, tube-like

formations between host cell surface and virus particle were visualized by TEM (**II,** Figs. S1 B, C). Such structures have been previously reported for icosahedral-tailless bacteriophages, e.g. PRD1, and are used for genome delivery (Peralta et al., 2013). The virion of HCIV-1 is icosahedral-tailless with an internal lipid membrane, enclosing a ~31 kb linear dsDNA genome (**II**). HCIV-1 acquires its lipids selectively from the host cytoplasmic membrane (**II,** Fig. 3). HCIV-1 is structurally and genetically similar to the other haloarchaeal icosahedral-tailless internal membrane-containing viruses, SH1, PH1, and HHIV-2, which have been classified to *Alphasphaerolipovirus* genus (**II**) (Pawlowski et al., 2014). HCIV-1 genome regions that encode major capsid proteins and a putative packaging ATPase are highly similar with those of the other alphasphaerolipoviruses (over 90% amino acid sequence similarity). On the contrary, the spike protein sequences are the least conserved in HCIV-1 and alphasphaerolipoviruses, highlighting the conservation of the virion core elements (Abrescia et al., 2012; Bamford et al., 2002). High amino acid similarities of the core structural proteins to those in the alphasphaerolipoviruses (**II,** Fig. 4, Table S2) (Bamford et al., 2005; Jaakkola et al., 2012; Porter et al., 2005) suggest that HCIV-1 virion assembly might resemble the model suggested for HHIV-2 (Gil-Carton et al., 2015).

The molecular characterization of HCIV-1 revealed that this virus displays many features in common with the other halophilic icosahedral-tailless membrane-containing viruses, HHIV-2, SH1 and PH1. These viruses are not identical but they share high similarity in the conserved genomic regions, i.e. those encoding MPCs and a putative ATPase (Fig. 4 C (II)). Very recently, our proposal to assign HCIV-1 to the *Alphasphaerolipovirus* genus of the *Sphaerolipoviridae* family has been approved by the ICTV. Notably, the current alphasphaerolipoviruses have been isolated from very distant geographical locations and at different times. Considering the enormous number of viruses in the virosphere one should not find closely related viruses unless the diversity is extremely limited.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis focused on the different mechanisms of archaeal virus exit. In this particular case, viruses SH1, HHTV-1, HCIV-1, His1 and His2 were used and new methods that can be utilized in high salinity conditions were developed. In order to better follow the virus-host interaction and virus exit events, the traditional turbidity and progeny virus release assays were supplemented with electrochemical real-time measurements and an ATP assay. This was needed to clarify the discrepancies in the literature regarding SH1 virus exit mechanism. This approach was also used during the overall description of the newly isolated HCIV-1 virus.

Virus exit was monitored concomitantly using multiple technologies which provided accurate information regarding virus life cycles. SH1, HHTV-1 and HCIV-1 virus exit caused a rapid drop of turbidity, allowed the binding of the lipophilic PCB anion, and caused a decrease in cellular respiration. All these events matched with the timing of the virus progeny release indicating that cell lysis is the mechanism for viral exit. Surprisingly, His1 and His2 caused hardly any physiological changes in the host cells during virus release. This indicates that these two viruses most probably use budding as their exit mechanism.

The methodology adapted and used here can be applied to liquid cell cultures or almost all cell types. The read-out of the results can be obtained on-line increasing the accuracy of the methods. In addition, the concomitant measurements make it easier to compare the obtained results.

6. ACKNOWLEDGEMENTS

This work was performed at the Programme on Molecular Virology, at the Institute of Biotechnology and Department of Biosciences, Faculty of Biological and Environmental Sciences, University of Helsinki, and supervised by Academy Professor Dennis Bamford and Docent Hanna Oksanen.

I owe my deepest gratitude to my supervisor Prof. Dennis Bamford for giving me an opportunity to join the well-adjusted collective in his lab as well as agreeing to be my supervisor. Thank you for adapting my PhD to my needs and believing in me even in most doubtful moments, it kept me going. You always would find time for a short discussion as well as a long meeting. Your patience and gentle approach encouraged me to be better.

I am sincerely grateful to my second supervisor Hanna Oksanen for her passion for excellence. Her advice was always on spot and her guidance was important for my successful growth as a scientist. Thank you for your care, time and patience to lead me through my PhD.

I owe my gratitude to Docent Benita Westerlund-Wikström and Docent David Fewer for agreeing to be my thesis follow-up members. Our meetings made me feel ensured that I am going towards the right direction and provided me with more confidence.

I am grateful to pre-examiners of this thesis, Docent David Fewer and Professor Paulo Tavares, for their time and effort to read my thesis, their professional insights and comments.

I would like to acknowledge Viikki Graduate School in Biosciences and Doctoral Program in Microbiology and Biotechnology for supporting my thesis project and providing needed study courses and events, also as funding the participation and traveling to international conferences abroad.

Likewise I want to acknowledge the support of employees and the use of experimental resources of EU ESFRI Instruct Centre for Virus and Macromolecular Complex Production (ICVIR) of the University of Helsinki, Finland.

I would like to thank Rimantas Daugelavičius for offering me to go to Finland for my internship and helping me to accommodate here, it was an important starting point for me.

Also thanks to Docent Janne Ravantti for scaring a soul out of me even before my coming to Finland, it made me more prepared for all challenges. Likewise I want to thank Doctor Nina Atanasova for always being there with words of encouragement and so needed answers to all small details about thesis writing requirements. You all, together with Tatiana Demina and Maija Pietilä, I thank for a great mutual work and respectable publications resulting out of it.

I am grateful to all members of DB-lab for creating such a welcoming atmosphere for me since I was a newcomer. You always were so supportive in giving professional advices or hand of help when I was in need. I want to express a special thanks my former and present co-workers who became my dear friends Tatiana Demina, Daria Starkova, Irma Orentaitė and Dr. Xiaoyu Sun, with whom I shared my life stories, feelings, hopes and despairs. Thank you for patiently listening, rejoicing and comforting me. It made me feel like at home.

Also I want to thank my family and friends outside my work. Thank you for supporting me on the days of self-doubt and helping me to manage my life outside of work. Many thanks for Bohdana Rovenko for being a great friend and a flat mate, you always was close to me as a sister. Sophia Albov, thank you for proving me that distance is not an obstacle for a heartfelt friendship, you showed me the world.

Julija

Helsinki, December 2016

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Helsinki 2016 ISSN 2342-5423 ISBN 978-951-51-2710-5