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Matti Jalasvuori

Viruses Are Ancient Parasites that Have Influenced the Evolution of Contemporary and Archaic Forms of Life

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Ymmärrän aineen synnyn, sen hitaan kiertokulun alkuräjähdyksestä tähtiin ja eläviin olentoihin, mutta siinä ei ole kaikki. Aina välillä on hyvä polvistua vesirajaan ja katsella merta sekä tähtiä, outoja ajatuksia mielessään liikutellen. Ei kannata uskoa mihinkään, mikä ei ole totta, mutta ei pidä myöskään olla tunteita vailla oleva yksinkertainen kone, jonka elämän täyttämiseen riittää pelkkä tosiasioiden, havaintojen ja mittausten loputon jono. Välillä on hyvä ottaa kosketusta johonkin, mikä ei oikein tahdo mahtua ihmisen tajuntaan.

Hiekka, aallot, tähdet. Kaikkeus.

Esko Valtaoja, Ihmeitä

ABSTRACT

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Yhteenveto: Virukset ovat muinaisia loisia, jotka ovat vaikuttaneet nykyisten ja varhaisten elämänmuotojen kehitykseen
Diss.

Even the simplest bacterial cell is a very complex organism that could not have emerged spontaneously within the lifetime of the Universe. Therefore the first cells were already products of evolution. However, very little is known of this primordial evolutionary process that produced the cellular organisms. Interestingly, viruses appear to have emerged before the first bacterial and archaeal cells and thus the process seem to have generated also viruses along with the cells. In this thesis a new lineage of ancient viruses (infecting solely thermophilic bacteria and halophilic archaea) was discovered. This lineage is evolutionarily related to a previously studied lineage of double-beta barrel viruses and therefore these icosahedral, inner membrane containing viruses probably separated into two lineages already before the emergence of the last universal common ancestor of cells. In order to study the possible processes that could have generated first viral genes within a primordial community of simple (RNA-based) replicators, a rule-based computing system was developed. Computational simulations suggested that the first viral-genes may have served as an information sharing mechanism between proto-cells. Possibly the modern viruses emerged from these proto-viruses while the cells evolved into more complex entities. Viruses could also have been the driving force in the isolation of the proto-cells from the primordial community since the isolation would have made the emerging cells less likely to be infected with viruses. In order to test this hypothesis, the effect of genetic isolation was studied with modern bacteria under bacteriophage selection. Bacteriophages, which exploit the gene-transfer apparatus of bacteria for recognizing the host cell, rapidly decreased the percentage of bacteria that are able to exchange genetic material with other cells. This suggests that similar isolation might indeed have occurred also on primordial conditions due to viral selection.

Keywords: viruses; plasmids; origin of cells; bacteriophages; astrobiology; Bacteria; Archaea.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred in the text as following Roman numerals.

I Jalasvuori M., Jaatinen S.T., Laurinavicius S., Ahola-Iivarinen E., Kalkkinen N., Bamford D.H. and Bamford J.K.H. (2009). The Closest Relatives of Icosahedral Viruses of Thermophilic Bacteria Are among the Halophilic Archaea. J Virol. 83:9388-97.

II Jalasvuori M., Pawlowski A. and Bamford J.K.H. (2010). A Unique Group of Virus-Related Genome Integrating Elements Found Solely in the Bacterial Family Thermaceae and the Archaeal Family Halobacteriaceae. J Bact. 192:3231-3234.

III Sozhamannan S., McKinstry M., Lentz S.M., Jalasvuori M., McAfee F., Smith A., Dabbs J., Ackermann H.W., Bamford J.K.H., Mateczun A., Read T.D. (2008). Molecular Characterization of a Variant of Bacillus anthracis Specific Phage AP50 with Improved Bacteriolytic Activity. Appl Environ Microbiol. 74:6792-6.

IV Jalasvuori M., Friman V., Nieminen A., Bamford J.K.H., Buckling A. Bacteriophages can effectively reduce the plasmid-borne antibiotic resistance in bacterial populations. Submitted manuscript.

V Friman V., Hiltunen T., Jalasvuori M., Lindstedt C., Laanto E., Örmälä A-M., Laakso J., Mappes J., Bamford J.K.H. High temperature and parasitic bacteriophages indirectly select bacterial virulence in free-living environment. Submitted manuscript.

VI Jalasvuori M., Jalasvuori M.P. and Bamford J.K.H. (2010). Dynamics of a Laterally Evolving Community of Ribozyme-like Agents as Studied with a Rule-based Computing System. Orig Life Evol Biosph. 40:319-334.

VII Jalasvuori M. and Bamford, J.K.H. (2008). Structural Co-Evolution of Viruses and Cells in the Primordial World. Orig Life Evol Biosph. 38:165–181.

VIII Jalasvuori M. and Bamford, J.K.H. (2009). Did the Ancient Crenarchaeal Viruses from the Dawn of Life Survive Exceptionally Well the Eons of Meteorite Bombardment? Astrobiology. 9:131-137.

IX Jalasvuori M., Örmälä A-M., and Bamford, J.K.H. (2009). On the astrobiological relevance of viruses in extraterrestrial ecosystems. Int J Astrobiol. 8:95-100.

My contributions to the original publications:

I I annotated and analyzed the genomes of P23-77 and IN93, identified the related elements among Haloarchaeal species, wrote most of the paper and prepared most of the figures.

II I annotated and analyzed the genomes of IHP, MeioSilP1, MeioRubP1, HaloMukP1 and HaloMukP2, prepared the figures and wrote the paper.

III I annotated and analyzed the genome of AP50 and wrote part of the paper.

IV I came up with the hypothesis, designed the experiments, executed about half of them and analyzed the results. I wrote the paper together with Ville Friman and Angus Buckling.

V I participated into the experimental design of the study. I isolated the novel viruses, helped analyze the results and wrote part of the manuscript.

VI I designed and programmed the computational system, performed all the experiments, analyzed the results, prepared the figures and wrote the paper.

VII I made the hypotheses and wrote the paper.

VIII I made the hypotheses and wrote the paper.

IX I designed the experiments, performed half of them and wrote the paper.

ABBREVIATIONS

ATP	Adenosine triphosphate
bp	base pair (DNA)
CNI	Close-Neightbour Interchange
C-terminus	Carboxyl terminus (protein)
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
ER	Endoplasmic Reticulum
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
Inc	Incompatibility type
ITR	Inverted terminal repeat
LHB	Late Heavy Bombardment
lMCP	large major capsid protein
LUCA	Last Universal Common Ancestor
MCP	Major capsid protein
ME	Minimum Evolution
mRNA	messenger RNA
PSK	Post segregational killing
RNA	Ribonucleic acid
SARS	Severe Acute Respiratory Syndrome
SIV	Simian immunodeficiency virus
S-layer	Surface layer
sMCP	small major capsid protein
STIV	Sulfolobus Turreted Icosahedral Virus
tRNA	transfer RNA
T-number	Triangulation number
VP	Virus Protein
Å	Ångström (unit)

1 INTRODUCTION

It was Robert Hooke's discovery on the year 1665 that suggested some of the living organisms to comprise tiny cellular structures. Few years later Antonie Van Leeuwenhoek observed first cells under the microscope. Theodor Schwann, Matthias Jakob Schleiden and Rudolf Virchow formalized the current knowledge on cells and developed the so-called cell theory in the 19th century. The theory states that all life is formed of cells, which divide in order to reproduce. Ever since the cellular view of life has been the dominating one among life scientists. However, cell theory has some deficiencies, and thus it does not provide a thorough model for understanding all aspects of life. According to the theory, cells always arise from pre-existing cells. The very first life forms did not consist of cells in the same sense as modern organisms, thus at some point on primordial Earth the first true cells must have emerged from something more primitive. Moreover, viruses have a multitude of features that could account for a living organism but they do not fit exactly into the cell theory. Viruses evolve, reproduce genetically and can go extinct. Interestingly, attempts to patch these two shortcomings of the cell theory by combining viruses and the origin of first cells might provide novel insights to our understanding of life.

There are some 10^{32} viruses on Earth. Viruses have forced innumerable cellular organisms to their deaths by hijacking the cellular machinery for virus production. Some studies suggest that there occur about 10^{26} virus infections every minute (Pedulla et al. 2003). Ten million billion billion cells are invaded by a virus every second. If all cells weighted only the mass of an average bacterium, ~10^{-12} g, and only one tenth of the infected cells would actually die due to the infection, then billion kilograms of cells would be slain by viruses every single second. In other words, the amount of cells equal to whole mankind in biomass would be destroyed every minute – in a year the viral annihilation would consume the mankind 525 948 times. And this massacre has been going on for the past four billion years (Hendrix et al. 1999).

Choose a genetically reproducing entity randomly from the biosphere and you would get ten viruses before catching the first cell. Some studies have

suggested that a great portion of genetic information resides in virus genomes and thus significant fraction of the diversity of genes is located outside of cells (Angly et al. 2006). The cellular life is surrounded by a rapidly evolving and genetically unique viral halo that constantly checks our ability to combat viral infections.

In the past decades our view of the biosphere has been expanding as our knowledge on viruses has increased. Yet many questions remain. How massive role do viruses play in the life on Earth? Were virus-like organisms already driving the evolution of archaic entities within the primordial hatchery of life? Do we even realize all the possible ways by which viruses might affect the evolution of life? In this thesis I address some of these questions.

I identify a novel viral lineage that appears to have emerged before the last universal common ancestor (LUCA) of cells. Moreover, I show that these viruses share similarities with a previously established double beta-barrel capsid protein viral lineage. The two virus-types might have separated into two lineages before the emergence of modern Bacteria and Archaea, suggesting that the primordial community of life was sufficiently big and long lasting for viruses to diverge. Furthermore, I argue that the extremely diverse crenarchaeal viruses might be well-preserved remnants of the early virosphere. The potential role of the first virus-like capsid genes in a primordial compartment matrix was studied by simulating the evolution within a population of ribozyme-like agents. These genes appear to mediate the distribution of innovation between emerging proto-cells. I also hypothesize that some of the very first viruses might have promoted the genetic isolation of the first independent cellular organisms. In line with these arguments is our demonstration that the bacterial sex-organs were highly unfavorable to be maintained in presence of conjugative plasmid-dependent bacteriophages. Moreover, presence of bacteriophages appears to hinder the evolution of virulence-traits in some bacteria. Bacteriophages could also be used to reduce and restrict the spread of antibiotic resistances. In the end I discuss some potential ways by which viruses might help us to approach the hypothetical life elsewhere in the Universe. In this chapter I focus on introducing the reader to some of the relevant literature covering viruses, cells and early life.

1.1 An overview to prokaryotes: Bacteria and Archaea

Life on Earth consists of three primary cellular types, Bacteria, Archaea and Eukarya. Prokaryotes comprise the first two kingdoms of life: Bacteria and Archaea (Woese & Fox 1977). This thesis covers mostly prokaryotes and their viruses. However, emergence of some unique features of the third domain, Eukarya, is also discussed.

Cell is a biological unit that is surrounded by a cellular membrane. There are fundamental differences in each of the three cell types, but also significant similarities. For example the bacterial and eukaryal cells use similar fatty acid

membranes composed of glycerol-ester lipids. The isoprenoid membrane of Archaeal cell instead is build out of glycerol-ether lipids. Archaeal membranes are also unique because of their stereochemistry as the glycerol-group is reversed in comparison to those of other organisms (Nishimura & Eguchi 2007). However, in many other features Archaea is more similar to Eukaryotes than Eukaryotes are to Bacteria. Indeed, phylogenetic analyses have exhaustively suggested that Bacteria were the first to diverge from the Archaea-Eukarya branch of life (Woese 1998) (but also other divergence histories have been suggested, see e.g. Gupta 2001).

Prokaryal cells are often surrounded with cell walls that help the cell to maintain the structure under osmotic pressure, prevent macromolecules from reaching the cell membrane and act as a physical and chemical barrier between the cell and the environment. Bacterial cell wall components (the peptidoglycan molecules) are synthesized within the cell and then transferred outside (Higashi et al. 1967). Gram-positive bacteria have a thick murein surrounding the cellular membrane whereas Gram-negative bacteria have a thinner murein layer that is surrouded on both sides by membranes (Mudd et al. 1941). The outer membrane of Gram-negative bacteria contains also glycolipids and lipoproteins (Schröder et al. 2008). Archaea have various cell wall types in comparison to the single type (peptidoglycan) in Bacteria. Most archaeal walls are assembled of surface-layer proteins that form a rigid web, the so-called S-layer, around the cell (Engelhardt 2007). However, archaeal *Methanobacteriales* do not use the S-layer type of a cell wall but instead they employ a peptidoglycan-like wall in which the chemistry of the peptidoglycan molecules is, however, somewhat different from the bacterial version (White 1995).

Genetic information of all organisms is present in an information collective called the genome. Each gene encodes a protein or a biologically active RNA polymer that is responsible for carrying out an enzymatic function or structural task in the cell. Genomes may also contain, sometimes very large, regions of DNA between genes. However, only very recently this so-called junk DNA has been realized to play an important part in the life of organisms (Zuckerkandl & Cavalli 2007). Yet, junk DNA is much more common in eukaryal genomes than in prokaryal chromosomes. Archaeal and bacterial genomes are usually circular and consist of a single chromosome whereas eukaryal chromosomes are linear and often multiple in number. The replication of prokaryote genomes starts at one position. However, in archaeal *Halobacterium* and *Sulfolobus* genuses the replication starts at two or three points of origin, respectively (Zhang & Zhang 2005). Bacterial chromosomes contain around 500 to 10000 genes and archaeal genomes have been observed to consist of ~500 to 5000 genes (McCutcheon 2010). The parasitic bacterial and archaeal species that relay on the resources of other organisms are usually the ones with the small genomes. Large genomes instead belong mostly to those prokaryotes that survive in demanding and ever changing conditions.

1.2 Horizontal gene transfer

Horizontal gene transfer (HGT) refers to a situation in which a given gene appears to have been transferred from one cellular organism to another laterally rather than being vertically inherited from the parent(s). In other words, HGT accords a case of non-darwinian evolution where a gene (and thus the the potentially expressed phenotypic trait) is not present in the parents but instead the gene (and the trait) has been acquired from an external source. There are three different observations that can be explained most plausible with HGT (Doolittle 1999a). Firstly, sometimes the phylogenetic history of individual genes in a given genome does not follow the ribosomal RNA tree of life. This indicates a recent event of gene addition to the particular genome. Secondly, some genes differ from the rest of the genes in the genome by their G + C content, codon-usage and gene order, suggesting that these genes and the rest of the genome have evolved separately (within the genome of a different entitity). Thirdly, certain genes in some genomes appear to be more closely related to homologues in distant rather than close relatives or such genes are absent in the close relatives altogether. These notions have lead to a view of life where organisms are a part of a phylogenetic network rather than a phylogenetic tree of life (Doolittle 1999b).

The potential event of HGT between two bacterial species depends often on the mutual compatibility of transferring agents between the two species. These include the specificity of bacteriophages, plasmid incompatibility and restriction modification systems (Binnewies et al. 2006). Moreover, the recently identified CRISPR-system appears to hinder the potential for horizontal gene transfer among (at least) *Staphylococcus* species (Marraffini et al. 2008). Some studies also suggest that HGT depends mainly on physical proximity rather than phylogenetic proximity of organisms (Matte-Taillez et al. 2002).

Bacterial species such as *Synechocystis* spp were reported to have acquired 17 % of its genes from foreign sources (Ochman et al. 2000). Comparison of five closely related *Chlamydia* genomes indicated that HGT played a significant role in the evolution of each of the particular bacterial species (Dufraigne et al. 2005, Ortutay et al. 2003). In 2006 Choi and Kim published a study of the global extent of horizontally transferred genetic material encoding specific protein domains (and not complete genes). They concluded that over 50% of Archaea, 30-50% of Bacteria and less than 10% of Eukarya had acquired at least one protein domain by HGT. However, Choi and Kim also noted that HGT would have only a small effect on the reliability of the generated phylogenetic histories if complete genomes or small-subunit ribosomal RNAs were used for the construction of the tree. Kurkland et al. 2003, similarly suggested that HGT clearly occurs but the traditional Darwinian view of the evolution of life is still valid. They argued that the assumption of the ubiquitous influence of HGT throughout evolution is exaggeration. However, such important features of cells as the DNA mitchmatch repair system appear to have been horizontally

transferred from a primordial bacterium to Eukarya and Archaea (Lin et al. 2007). Syvanen hypothesized in 2002 that the genetic code was not complete in the last common ancestor of cellular domains and some of the transfer RNA genes had been horizontally spread between the already established lineages. Moreover, for example, transfer RNA-methytransferases of Archaea appears to be of bacterial origin (Urbonavicius et al. 2008). Interestingly, these and other aspects suggest HGT to have been much more frequent and more significant right after the emergence of life on Earth (Woese 1998, Woese 2000, Woese 2002).

In this thesis I demonstrate, to my knowledge, the first case where bacteriophages hinder the HGT between bacterial species (IV). These results could help explain why the role of HGT has been relatively little in the evolution of contemporary organisms. Moreover, the potential role of virus-like genes in mediating HGT within the early evolutionary community is studied (VI, VII).

1.3 Conjugative plasmids

There may be independently replicating genes located outside of the cellular chromosome in bacterial and archaeal cells. These genes belong to either plasmids or viruses. Viruses are reviewed later in this chapter and thus I focus here on the conjugative (IncP-type) plasmids. Conjugative plasmids are plasmids that are able to force their host cells to transfer genetic material (and thus the plasmid itself) between bacterial cells through conjugation (Amabile-Cuevas & Chicurel 1992). All plasmids fall into one of the many incompatibility (Inc) groups. If two plasmids belong into the same group, they cannot be stably maintained within a single cellular line. IncP-1 plasmids are able to replicate in majority of Gram-negative bacterial cells and they can be transferred to Gram-positive bacteria and even to mammalian cell lines (Waters 2001). IncP-group is an example of self-transmissible, stably replicating and wide host-range plasmids that appear to play important role in the evolution of bacterial communities (Adamczyk & Jagura-Burdzy 2003). They replicate using a theta replication mode. Some host proteins, such as DNA polymerase III holoenzyme, are required for the IncP-1 plasmid replication. Plasmids might also employ somewhat different replication strategies depending on the host cell (Doran et al. 1999). The copy number of IncP-1 plasmids is around 5 to 7 per bacterial chromosome. Tight control of certain operons (e.g. *trfA*) is responsible of maintaining the copy number. The stable maintenance and distribution of the plasmids to both daughter cells is in many respects similar to the maintenance and partitioning of the host chromosomes (Bignell & Thomas 2001). IncP-1α type plasmids also rely on a postsegregational killing (PSK) mechanism for ensuring the distribution of the plasmid to both cells during cell fission. The PSK mechanism is based on a toxin-antitoxin principle that destroys plasmid-

free cells (Roberts & Helinski 1992). The plasmid produces two agents, one being a relatively stable toxin molecule and an unstable antidote for the toxin (preventing the poisonous effect of the toxin). Both elements remain within the cells of plasmid free segregates and the more stable toxin eventually causes the cell death because the antidote is no longer produced. IncP-1 plasmids also encode a mating pair formation apparatus, which is responsible for recognition and transfer of the plasmid to susceptible bacteria (for review, see Adamczyk & Jagura-Burdzy 2003). The apparatus includes a sex-pilus that bridges the two cells together. Pilus is composed of 78 amino acid long proteins arranged into a cyclic ring-like structure (Eisenbrandt et al. 1999). The plasmid is transferred through the pilus in a single stranded form during rolling circle replication. The complementary strand is synthesized within the recipient cell.

Conjugative plasmids have an important role in the evolution of the host cell. They spread quickly in heterogeneous bacterial populations (Dionisio et al. 2002). Some plasmids can also recombine into the host chromosome and reside there similarly with genome integrating viruses (Mei et al. 2007). Conjugative plasmids and conjugative transposons are often responsible for the emergence of antibiotic resistance in clinically important bacteria (Bennett 2008). They also appear to be abundant in multi-resistant bacteria isolated from soil samples and thus these environments might serve as a supply of antibiotic-resistance genes for emerging drug-resistant pathogens (Ansari et al. 2008). The route of transfer of antibiotic resistance genes may occur from bacteria of livestock animals to human pathogens (Gebreyes & Thakur 2005). In 2008 Hradecka et al. tested 23 antibiotic resistant field isolates of *Salmonella* to find out whether the resistance is encoded by a transferrable conjugative plasmid. Twelve of the isolates were able to spread the resistance to non-resistant cells. Kim and colleagues sequenced two IncJ-type plasmids that were isolated from fish pathogen *Photobacterium*. Their analysis suggested that conjugative transfer regions and drug resistance regions were the most diverse ones in the otherwise conserved plasmids (Kim et al. 2008). In 2008 Tamang and colleagues studied resistant clinical *Enterobacteriaceae* isolates from Korean hospitals and noticed multi-resistant species to often harbor conjugative plasmids.

It appears that conjugative elements are very often the reason behind drug resistant phenotypes in any given environment. In this thesis I demonstrate a novel way for forcing the bacterial cells to lose IncP- and IncN-type conjugative plasmids (IV).

1.4 Prokaryotic viruses and viral life strategies

Viruses are common obligate parasites of cells. They are inactive outside of their host cells (excluding one peculiar *Crenarchaea* infecting virus, ATV, which develops its tail structure in extracellular environment, Haring et al. 2005). Infectious virus particle is known as virion. Virion comprises a protein capsule, i.e. capsid, which encloses the virus genome. Viruses can also have lipids and

other molecules associated with the virion, but only the proteins and the nucleic acids are exclusively ubiquitous features of viruses. Viruses differ fundamentally from all cellular entities in one respect. Virus progeny is always assembled from individual particles whereas cells always divide to reproduce. This is why viruses do not fit into the cellular theory. Studies concerning virus abundance suggest that there are ten viruses for every cell in the biosphere (Bergh et al. 1989, Borsheim et al. 1990). This means that the virosphere may comprise up to 10^{32} viruses – making them the most prevalent entities on Earth.

There are great deviations between different types of viruses, but they all still employ the same basic strategy for reproduction. Most (complete) virus life cycles begin when the virus recognizes the cellular receptor (Poranen et al. 2002). The attachment of the virus to the host is followed by the introduction of the complete or partial virion or just the virus genome into the cell. Plant viruses, however, often penetrate into the plant cells through vectors, such as insect bites, and thus no virus mediated recognition of the host cell occurs (Villarreal 2005). Within the cell, the virus uses the host machinery for producing and assembling new virus particles. Some viruses integrate into the host chromosome as a part of their life cycle (Holzel & Sokol 1974, Doerfler 1975). When new virions are ready, the next generation of infectious virus particles escapes the cell to infect new hosts. However, there are two major forms of virus life strategies that arguably have very different impact on their hosts in evolutionary sense (Villarreal 2005). The life strategy, which starts from the recognition of the host cell and ends into the destruction of the host cell, is called lytic or virulent. The other strategy is called lysogenic or temperate and it does not involve host cell destruction. Instead, during lysogeny virus exists within the host in a stable and, in a sense, peaceful state. Lysogenic cycle is only a little burden to the cell and lysogenized host is generally immune to further infections by that virus type. Lysogenic viruses may, however, switch into the lytic strategy and produce virions and cause the lysis of the host cell. Some prokaryal viruses (and many eukaryal ones) can also produce virions without destructing the host cell (Villarreal 2005). Some plant viruses also travel between cells through the cell-wall traversing plasmodesmata.

Bacteriophages, or just phages, are viruses infecting bacteria. Bacteriophages use two very different strategies to exit from the host cells (Young 1992). In one, bacteriophage encodes holins that first creates holes into the membranes. The holin-channel is then used by lysins that pass through the membrane to digest the cell wall (see e.g. Krupovic et al. 2007). As the cell wall fractures, the internal pressure of the bacterium causes the cell explosion. In the other strategy, the bacteriophage encodes proteins that activate cellular autolysis.

It has been shown for bacteriophages that the sizes of phage-formed plaques on agar-plates can be increased by selecting phages from the edges of the plaque for subsequent round of plaque assays (Abendon & Culler 2007). This suggests rapid evolution in the lysis timing and infectivity of the phage. Delbruck noticed already in 1945 that it appears to be irrelevant whether a

bacterial cell is infected with one virus or "superinfected" with several viruses. Thus, for viruses to efficiently produce progeny and therefore infect the maximum potential amount of cells, it is not optimal always to attach to the first encountered cell. Moreover, the timing of lysis is directly related to the virus fitness, as the optimally spreading virus needs to retain from lysing the cell either too early or too late (Wang 2006). In other words, virus fitness does not always correlate with its killing capacity (Herrera et al. 2007).

The control of lysis can be very complicated. MS2 virus, for example, expresses the lysin genes if a frame shift event occurs upstream of the translation of the coat protein mRNA (Kastelein et al. 1982). However, even strictly virulent phages, like phi29 infecting *Bacillus subtilis*, may retain from lysing the cell if the host is forming an endospore (Meijer et al. 2005). Most of the bacteriophage genes that are immediately expressed after infection influence bacterial proteins (causing inhibition, activation or redirection of the activity of the host proteins) in order to safeguard the bacteriophage from host defenses (Roucourt & Lavigne 2009). In case of phi29, the phage recognizes the phase of host life cycle and activates the life strategy accordingly. Along with virulent life-style, lysogeny is also a prevalent strategy in nature. In marine environments, for example, lysogenic life strategies are very common among phages. Jiang and colleagues isolated several phages from the coast of Hawaii and showed that all of these phages lysogenized their hosts isolated from the same samples (Jiang et al. 1998). Similar results have been acquired from various soil environments (Williamson et al. 2007).

In this thesis I analyze the genome of a virulent phage P23-77 and the genomes of multiple close relatives of P23-77 that are temperate viruses (I, II). Moreover, the mutations that resulted *Bacillus anthracis* virus AP50 to turn from being a lysogenic virus to a virulent one were determined (III).

1.4.1 Virus classification

There are several ways to classify viruses. In 1940s established Holmes classification puts viruses into just three groups. Group I contains viruses that attack bacteria (*Phaginae*). Group II comprises viruses that infect plants (*Phytophaginae*) and Group III comprises viruses of animals (*Zoophaginae*). In one of the other approaches of virus classification viruses are grouped according to the type of the genetic material and to the relationship of the genetic material to messenger RNA (mRNA) (Fauquet et al. 2005). David Baltimore, a Nobel Laureate, developed the so-called Baltimore classification, in which every virus belongs to one of the seven classes or groups (Fig. 1). Class I contains viruses with double stranded DNA genomes. The genes of Class I viruses can be transcribed readily by using the (-) sense strand as a template for RNA polymerase. Class II viruses have a single stranded DNA genome in the (+) sense form. The (+) sense strand is the coding strand of DNA, indicating that the genes cannot be transcribed directly into mRNA since the required template for RNA synthesis is absent. Thus Class II viruses have a double stranded DNA intermediate during the virus life cycle. Class III comprises viruses with double

stranded RNA genome. The (-) sense strand of Class III viruses is used to transcribe (+) sense strand, which is then used as an mRNA. Class IV viruses have a single stranded RNA genome, which can directly serve as an mRNA. Class V viruses have a single stranded RNA genome in the (-) sense form, requiring the synthesis of the opposite strand before the ribosome can use the RNA to encode proteins. Class VI viruses have single stranded RNA genome in the (+) sense form. These viruses are so called retroviruses. They have a DNA intermediate during the virus replication cycle, which is used for a template in the synthesis of mRNA. Class VII viruses have a double stranded DNA genome that replicates with a RNA intermediate.

Viruses belonging to a same class in Baltimore classification may not reflect phylogenetic relationship. However, as our knowledge on viruses has increased, Bamford has suggested that it should be possible to organize the vast virosphere into a reasonably few, phylogenetically related viral lineages (Bamford 2003). The viruses of these lineages share a common innate "self", which is responsible for the genome packaging and structural principles of the virion. The "self" would retain its identity throughout evolutionary times while the "non-self" components of the virus genomes could be swapped with other viruses. Interestingly, the "self" approach can indeed link viruses belonging to different groups in the Baltimore system. The ssDNA virus HRPV-1 and the dsDNA virus His2 share multiple core genes (including the genes for major strcutural proteins and ATPases) but are members of Baltimore Groups I and II, respectively (Pietilä et al. 2009).

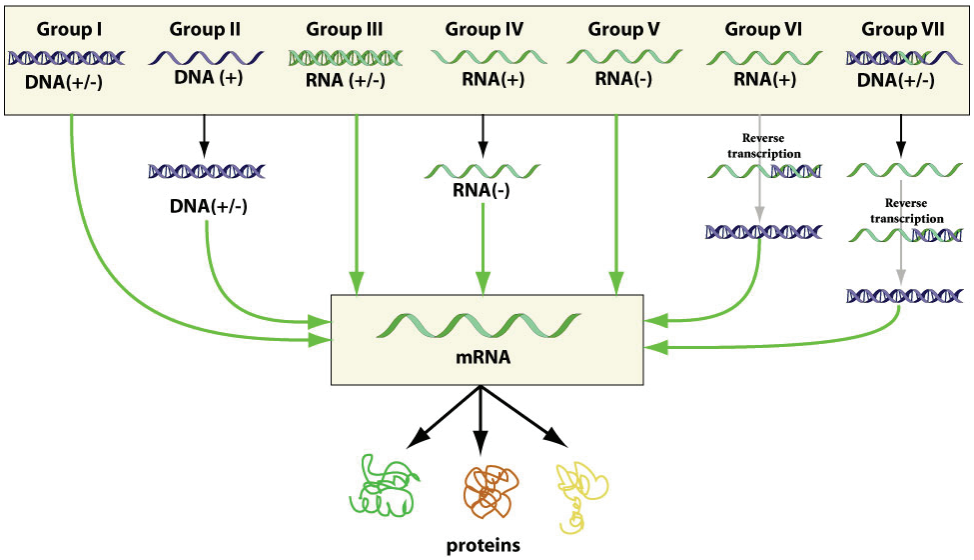

FIGURE 1 The Baltimore classification of viruses is based on the chemistry of virus genetic material and the relationship of the genome to the messenger RNA. Adapted with permission from ViralZone, Swiss Institute of Bioinformatics.

1.4.2 Bacteriophages - *Caudovirales*

Bacteriophages are viruses of bacteria. About 96 % of all currently identified bacteriophages belong to order *Caudovirales*, a group of phages with a head-tail morphology (Bradley 1965, Proctor 1997, Ackermann 2007). Their dsDNA genome is packaged by using molecular motors into an icosahedral protein capsid, called the head (Ackermann 2003, Letellier et al. 2004). A connector to this head-particle attaches the tail, which is responsible for host recognition. Due to the structural similarities, all tailed phages are considered to share a common origin (Effantin et al. 2006). However, *Caudovirales* is further divided into three distinct virus families, *Myoviridae*, *Siphoviridae* and *Podoviridae*, according to the differences in the tails (Ackermann 1998). *Myoviridae* consists of phages with long, contractile tails. Members of *Siphoviridae* have long and non-contractile tails. The tails of *Podoviridae* are very short. Lavigne et al. 2008, divided *Podoviridae* into two subfamilies called *Autographivirinae* and *Picovirinae*. The former subfamily contains the T7-like phages and the latter comprises phi29-related viruses. In 2009 Lavigne and colleagues unified also the classical and genomic classification of *Myoviridae*. They divided *Myoviridae* into three subfamilies: *Peduovirinae, Teequatrovirinae, Spounavirinae* and eight new independent genera.

One of the best-characterized members of *Myoviridae* is the *Escherichia coli* phage T4. T4-like phage genomes are very long for a phage, being around ~160-250 kbp of dsDNA (Nolan et al. 2006). Tail fibers of T4 are responsible for attaching the virion to the host cell. The tail is used as a hollow channel for translocation of the viral genomic DNA into the cell. T4 is a lytic phage, incapable for lysogenic life cycle. T4 arrests the host gene expression immediately after entering the cell. Its genome encodes many host-like functions such as genes for nucleotide metabolism machinery (Miller et al. 2003). T4-like phages appear to have acquired their genes from a highly divergent pool of genes in the microbial world and some of their genes have not been discovered from any other entity (Nolan et al. 2006). T4 also has eukaryal-like introns.

Members of *Podoviridae* have only a short tail. A well-known example of such a virus is the *Escherichia coli* phage T7 (see e.g. Agirrezabala et al. 2007). It is a strictly lytic phage with a genome of 40 kbp of dsDNA. T7 has an icosahedral capsid about 55 nm in diameter. One of the icosahedral vertices has a connector protein, which serves as a portal for the movement of the genomic DNA in and out of the capsid (Steven & Trus 1986). The packaging of T7 DNA is well studied. The right end of the genomic DNA concatemer is selected and it serves as a starting point of the packaging of the DNA into the prohead. DNA packaging proceeds at 140 bp/s at 25 ^{o}C costing a single ATP every 1.8 bp of packaged DNA (Morita et al. 1993). When quarter of the genome has entered the capsid, the prohead undergoes its maturation expansion (Shibata et al. 1987). After the expansion, the tail assembles on the outside of the connector. Interestingly, 80% to 90% of the DNA molecules of some T7-like phages

("phiKMV-like viruses") contain nicks at specific positions in one of the strands (Kulakov et al. 2009). There is no function yet associated with the nicks. Moreover, T7 has been used in various evolutionary experiments (Bull & Molineaux 2008). In this thesis, a T7-relataed phage PPV, infecting *Serratia marcescens*, was used in a serial culture experiment (V).

The tails of viruses belonging to the family *Siphoviridae* are long and non-contractile. The capsids also vary within the family since in some of the viruses they are isometric and in others the capsids are prolated. The best-studied member of *Siphoviridae* is the *Escherichia coli* infecting phage Lambda. Lambda genome consists of a dsDNA molecule of 48.5 kbp in length (Echols & Murialdo 1978). Lambda is mainly a lytic phage. However, under certain conditions it may select a lysogenic pathway and integrate into the host genome (see e.g. Kotewicz et al. 1977). Lambda replicates along with the host chromosome, but under host stress conditions Lambda may activate again and start producing new virus particles (Osterhout et al. 2007). Details of Lambda life cycle are well known. It involves complex interactions with phage-encoded repressors that cooperatively bind the Lambda genome in order to force it into either of the two possible life cycles (Stayrook et al. 2008). T4, T7 and Lambda, have all played a major role in the development of molecular biology. The morphotypes of these phages are depicted in Fig. 2.

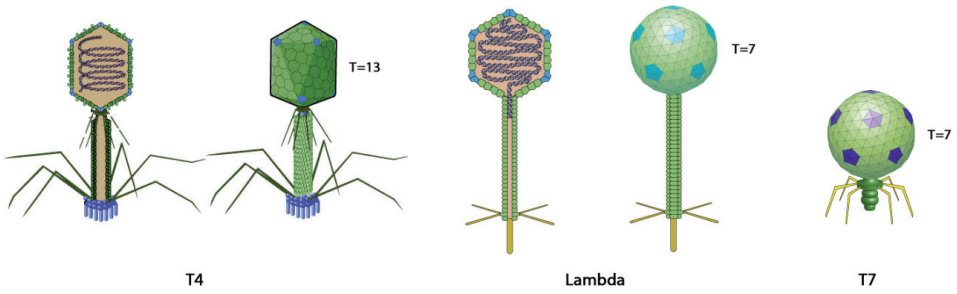

FIGURE 2 Morphological features of bacteriophages belonging to families *Myoviridae* (T4), *Siphoviridae* (Lambda) and *Podoviridae* (T7). T4 has a contractile tail. Lambda has a long, non-contractile tail. The tail of T7 is very short and non-contractile. The triangulation number, i.e. the organization of the capsomers in the icosahedrons according to the model developed by Caspar and Klug, 1962, of the phages is marked next to the head particle. The head of T4 is prolated. Adapted with permission from ViralZone, Swiss Institute of Bioinformatics.

1.4.3 Bacteriophages - *Tectivirales*

Family *Tectiviridae* comprises viruses with an icosahedral and tailless capsid. Beneath the capsid tectiviruses have a host-derived inner membrane (Olsen et al. 1974, Laurinavicius et al. 2004). The type member of *Tectiviridae* is a conjugative plasmid-dependent bacteriophage PRD1 (Fig. 3) (Olsen et al. 1974, Bradley & Rutherford 1975). PRD1 uses the mating pair complex encoded by

some conjugative plasmids of types IncP, IncN and IncW as a receptor. Expression of *TraF* gene of IncP-type plasmid RP4 was shown to be necessary for the virus attachment to the host cell (Kotilainen et al. 1993). Moreover, *TraF* mutants make the conjugative plasmid unable to conjugate and prevent PRD1 binding to the cell.

The genomes of tectiviruses are linear double stranded DNA of ~15 kb in lenght with covalently attached proteins at both ends. The terminal protein provides a hydroxyl group for priming the DNA synthesis during genome replication (Hsieh et al. 1987). The strand, which is not used as template, is displaced as the replication proceeds, eventually leading to a single stranded intermediate (Savilahti & Bamford 1986). Before the single stranded genome is completed into the double stranded form, the DNA probably folds into a spoon-like formation because the inverted terminal repeats at the ends of the genomes pair together (Gerendasy & Ito 1987). PRD1 encodes thirty-one genes, including a type B DNA polymerase (Savilahti et al. 1991). These genes are expressed in five operons (Bamford et al. 1991). The two early operons contain mainly genes for genome replication, including the genome terminal protein and the DNA polymerase (Savilahti & Bamford 1987). When the three late operons become active, the structural proteins required for producing complete virus particles and proteins responsible for genome packaging into the particle are expressed. The changes in host gene expression during PRD1 infection was studied, and it was noted that relatively little changes occurred before the virions were already assembled (Poranen et al. 2006). After virion assembly the level of host expression of stress inducible proteins was elevated. During PRD1 particle assembly free vesicles can not be observed, indicating that the virion lipid layer is acquired simultaneously with building of the protein capsid (Mindich et al. 1982). The genome of PRD1 is packaged through a single special vertex into the capsid (Strömsten et al. 2003, Gowen et al. 2003). PRD1 also requires chaperonins GroEL and GroES for correct protein folding (Hänninen et al. 1997). Moreover, PRD1 is a strictly virulent phage. It has no stable carrier form within the cell and PRD1 infection inevitably leads to destruction of the host cell. Life cycle of PRD1 takes around 50 - 60 minutes, but the length depends on the host cell, growth temperature and nutrient medium. Burst size of PRD1 is about one hundred virions when PRD1 infects *E. coli* growing in Luria-Bertani medium at 37 °C.

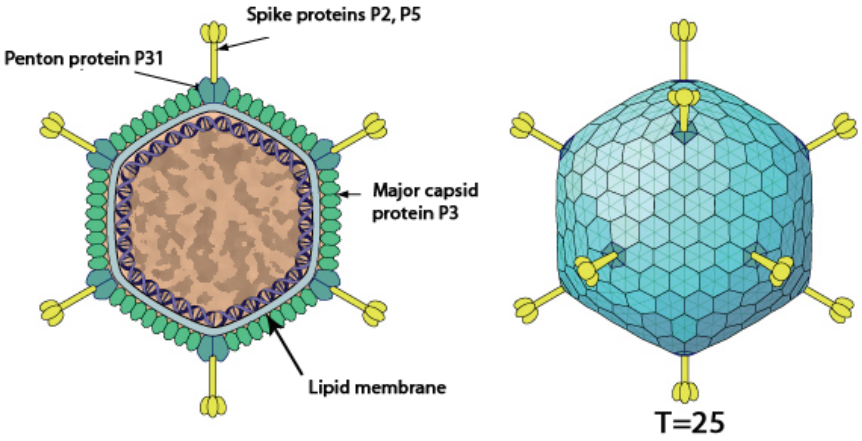

FIGURE 3 Schematic presentation of the tectivirus PRD1. PRD1 exploits triangulation number 25 (T=25) for capsomer arrangement. PRD1 has an inner lipid membrane beneath the protein capsid. The capsid is formed mostly of the major capsid protein P3. The vertex structure has a pentameric base, formed of protein P31, upon which the spikes, formed of proteins P2 and P5, are built. Adapted with permission from ViralZone, Swiss Institute of Bioinformatics.

Functions of several PRD1 proteins are known. In 1982 Bamford and Mindich demonstrated that proteins P3 and P5 form the polyhedral capsid. P5 protein was tested to be less sensitive to artificially introduced mutations than P3 (Huiskonen et al. 2003). P5 is directly connected to the penton base protein P31, upon which P5 and the receptor-binding protein P2 form two different spikes (Huiskonen et al. 2007). P32 is a minor membrane protein that appears to have a function in DNA delivery (Grahn et al. 2002). P16 is an integral membrane protein that stabilizes the adsorption vertex structure (Jaatinen et al. 2004). The vertex is extended by P20 and P22 to the virion lipid membrane (Strömsten et al. 2003). These two proteins are also necessary for binding of the packaging ATPase P9 via an accessory protein P6 to the virion. P35 encodes a holin protein, required in endolysin translocation to the cell wall (Ziedaite et al. 2005). Functionally defective P35 protein can be replaced with the Lambda-phage holin (Rydman & Bamford 2003). The details of the complete PRD1 virion were revealed as the whole virion crystal structure at 4 Å resolution was determined (Abrescia et al. 2004). The structure revealed the locations and orientations of proteins P3, P16, P30 and P31 in the virion. P30 was known to be a protein that stabilizes the capsid assembly (Rydman et al. 2001). Crystal structure of the virion revealed PRD1 to have sixty copies of P30, which acted as a tape-measure for determining the capsid size. N-termini of P30 form a hook that mediates dimerization of two P30 proteins. C-terminus sinks into the inner membrane at the 5-fold vertices of the PRD1 virion. P30 is elongated to follow the edges of four complete capsomers and the hook structure together with the inverted dimerization partner of P30 dimer elongates over the fifth facet. P30

dimer forms a string which reaches from one 5-fold vertice to another and this has been proposed to force PRD1 virion into a pseudo T=25 lattice. Domains of P30 and its position in PRD1 virion are presented in Fig. 4.

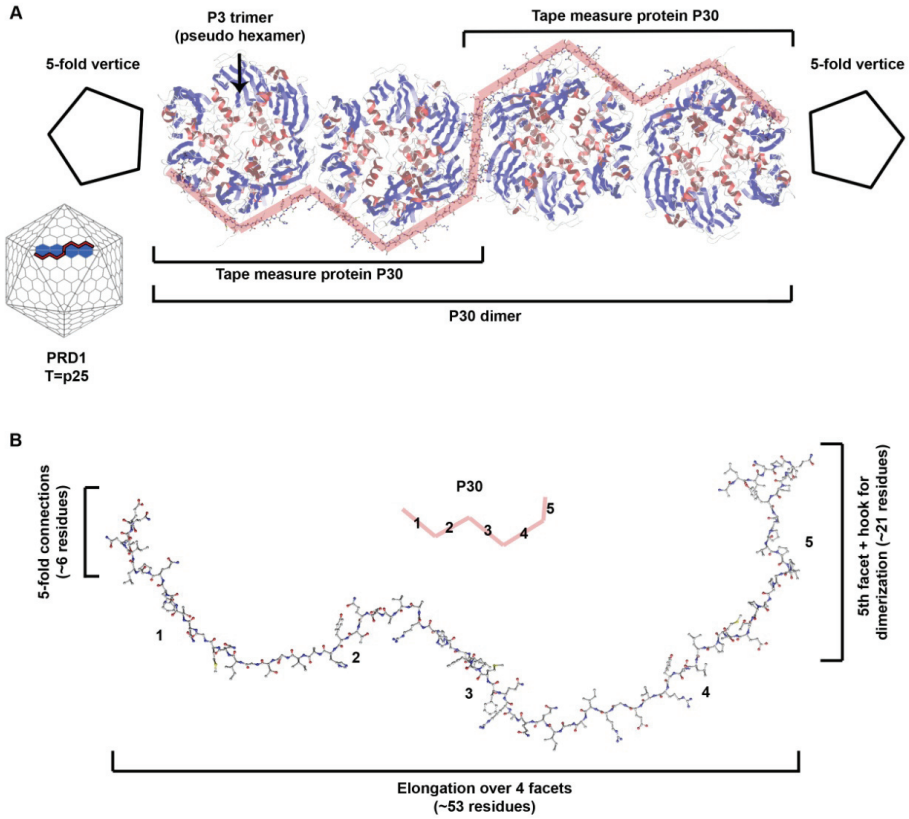

FIGURE 4 P30 is a suggested tape-measure protein in PRD1 virion. Position of P30 is marked with red line in the capsid model. P30 follows the capsomer edges as shown in panel A. There are distinct domains in P30 structure that either allow the protein to sink into the membrane at the vertexes, follow the capsomers or mediate the dimerization of two P30 proteins (panel B).

There are several close relatives to PRD1. PR3, PR4, PR5 and L17 are independent PRD1-like isolates that were shown to be structurally almost identical to one another (Bamford et al. 1981). Later on also their genome sequences were determined (Saren et al. 2005). Generally it has been argued that all bacteriophage isolates differ greatly from one another. However, interestingly these PRD1-like viruses share about 98 % identical genomes. Some other bacteriophages, like certain independent *Myoviridae* isolates, are also very similar to each other (Ceyssens et al. 2009), indicating that these bacteriophages are not taking part into the extensive recombination and gene swapping with other phages.

The other idenfied members of *Tectiviridae* are Gram-positive *Bacillus* infecting phages. Bam35 was first characterized in 1978 by Ackerman et al. to be a cubic *Bacillus thuringiensis* infecting bacteriophage with an icosahedral head and a tail-like structure that appears to be associated with the nucleic acid ejection. Bam35 is a lysogenic phage that is very similar to PRD1 under electron microscope. The tail-structure, which is formed by the extruding inner-membrane, is not present in stable, undisrupted virions (Fuller 2005). Bam35 infection starts from receptor binding and is followed by peptidoglycan penetration and interactions with the plasma membrane (Gaidelyte et al. 2006). The cell wall digestion function of Bam35 is associated with the virion. Bam35 genome is injected even into lysogenized hosts, but the late functions of the genome are suppressed (Gaidelyte et al. 2005). Moreover, it was demonstrated, for example, that specific proteins cause the lipid membrane curvature in Bam35 (Laurinmaki et al. 2005). The arrangement and length of PRD1 and Bam35 genomes are relatively similar, suggesting that their last common ancestor already had a similar genome (Ravantti et al. 2003). Bam35 is closely related to a linear plasmid pBClin15 (discovered from a *Bacillus cereus* strain, Strömsten et al. 2003) and to *Bacillus thuringiensis* phages GIL01 and GIL16 (Verheust et al. 2003, Verheust et al. 2005).

P23-77 is a *Thermus* infecting virus, which was first described and assigned into *Tectiviridae* by Yu et al. in 2006. P23-77 has an icosahedral capsid and contains an inner membrane (Fig. 5). The capsomers of P23-77 are decorated with two tower-like structures (Jaatinen et al. 2008). However, in this thesis it is shown that P23-77 fundamentally differs from the other members of *Tectiviridae* and therefore it is not a true member of the family.

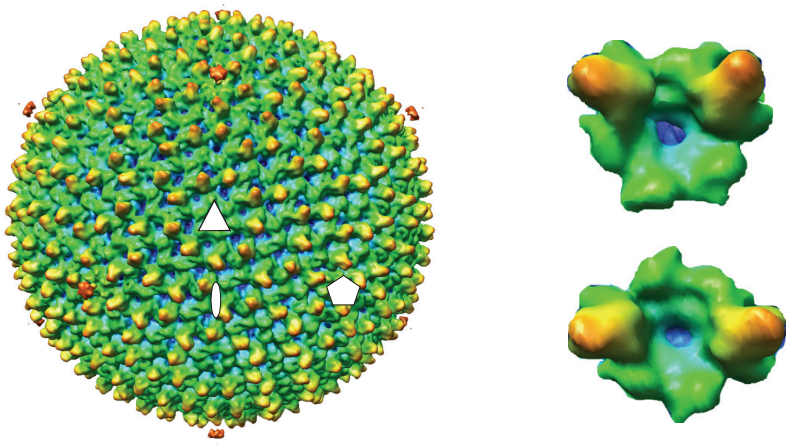

FIGURE 5 Reconstruction of a *Thermus* phage P23-77 capsid based on cryo-em data. The five-fold, three-fold and two-fold symmetry axes are marked over the virion. The capsomers are decorated with tower-like structures (depicted on right). Image source: Lotta Happonen and Sarah Butcher, University of Helsinki. Adapted with permission.

1.4.4 Other icosahedral bacteriophages with membranes

PM2 is a *Pseudomonas* infecting bacteriophage with similar morphology to PRD1 (Cota-Robles et al. 1968). PM2 is the sole member of the family *Corticoviridae*. PM2 uses the double beta-barrel major capsid protein to produce the T=21 arranged icosahedral virion (Abrescia et al. 2008). The novel lysis system of PM2 potentially includes a unique type of a holin (Krupovic et al. 2007). PM2 has a dsDNA circular genome, 10 kbp in length (Männistö et al. 1999). PRD1 and PM2 appear to be evolutionarily related due to the common double beta-barrel folds in the major capsid proteins and the shared motifs in the virus encoded ATPase (Abrescia et al. 2008). For a long time PM2 was the only identified member of the *Corticoviridae*. However, later it was demonstrated that PM2-like viruses are widespread in the genomes of marine bacteria. These integrated elements appear to have exchanged genes with various non-related viruses and other genetic elements (Krupovic & Bamford 2007). The PM2 virion is presented in Fig. 6a.

The *Pseudomonas* infecting RNA-virus phi6 also resembles tectiviruses in some respect. Phi6 forms an icosahedral capsid with no tail and contains a membrane (Bamford & Palva 1980). The virion structure is presented in Fig. 6b. However, the membrane envelope is above the protein capsid unlike in tectiviruses and P23-77. Phi6 genome is fragmented into three individual RNA-molecules. RNA viruses are often considered to form quasispecies structures where there is a cloud of sequences rather than a specific nucleotide order to determine certain virus (Bull et al. 2005, Holland 2006). Thus phi6, for one, has been used in studying the quasispecies evolution and mutational frequencies (Montville et al. 2005).

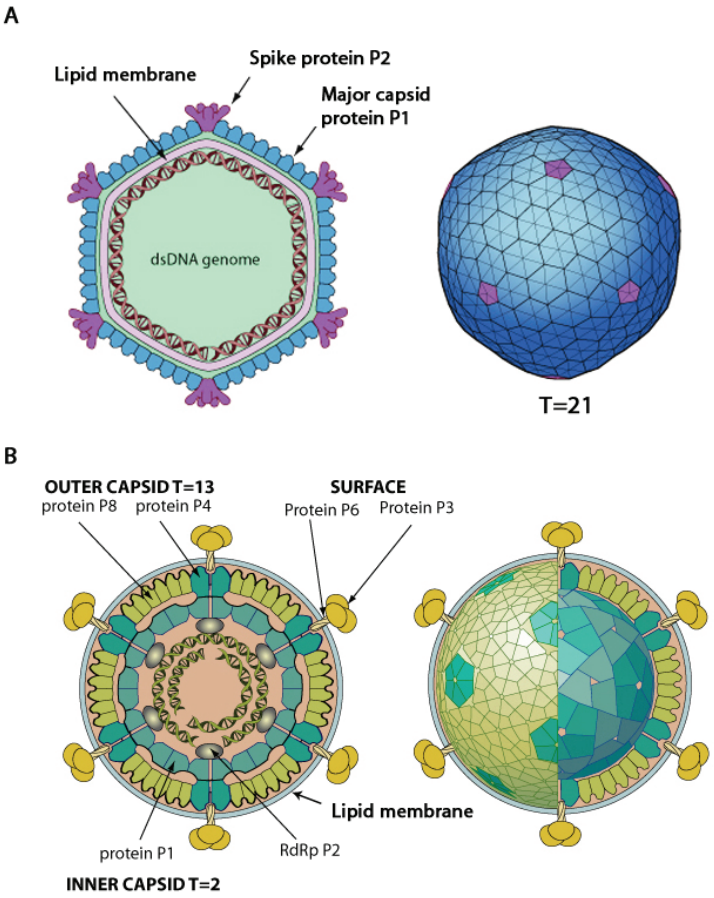

FIGURE 6 Schematic presentations of viruses (A) PM2 and (B) phi6. Both viruses form icosahedral capsid and have membranes as structural components. In PM2 the membrane is beneath the protein capsid whereas in phi6 the membrane is above the capsid. The locations of the main structural proteins are marked in the figure. PM2 uses triangulation number 21 (T=21) and phi6 uses triangulation number 2 (T=2) for the inner capsid and a triangulation number 13 (T=13) for the outer capsid. Adapted with permission from ViralZone, Swiss Institute of Bioinformatics.

1.4.5 Archaeal viruses and their unique morphotypes

Archaeal viruses are far less studied compared to bacteriophages. This is partly because bacteria were the first organisms to be used in studies that attempted to discover the basic genetics, molecular mechanisms and other functions of cells. Bacteriophages played a critical role for developing the basic knowledge of living systems. Nevertheless, the archaeal cells are also infected with large number of viruses.

Hypersaline lakes contain ten million virus-like particles in a millilitre of water (Dyall-Smith et al. 2003). At least some of these viruses infect

haloarchaeal cells belonging to genuses *Halomicrobium*, *Halobacterium* and *Haloarcula*. Among the morphotypes of the currently studied haloarchaeal viruses are the *Caudovirales*-like head-tail viruses, icosahedral viruses and pleomorphic viruses. Pietilä and colleagues demonstrated first evidence of a non-dsDNA archaeal virus (Pietilä et al. 2009). This single stranded DNA virus has a pleomorphic virion and it appears to be related to a pleomorphic bacterial virus infecting *Acholeplasma laidlawii*.

SH1 is an icosahedral virus with an inner membrane and a linear, dsDNA genome of ~31 kb in length (Bamford et al. 2007, Jäälinoja et al. 2008). It lytically infects euryarchaeal halophile *Haloarcula hispanica.* SH1 encodes an ATPase that has common domains with the ATPase of the double-beta barrel viruses (Krupovic & Bamford 2008). In this respect SH1 resembles the PRD1-type viruses. However, the capsomers of SH1 are decorated with tower-like structures, similarly with bacteriophage P23-77.

Crenarchaeal viruses are very diverse in their morphotypes (Prangishvili 2003). Rachel et al. for example, studied only two hot springs in Yellowstone National Park and were able to discover twelve clearly different viruses (Rachel et al. 2002). Among these and other studied morphotypes are rod-shaped helical particles, filamentous particles with thin terminuses, ellipsoid viruses with two long tails, spindle-shaped particles, short rod-shaped particles, filamentous particles with bulbous termini, filamentous particles with rounded ends, spindle-shaped particle with a helical tail, zipper-shaped particles, pleomorphic particles with arrow-shaped heads, spherical particles, droplet shaped particles, bottle shaped particles and icosahedral particles with turrets (Prangishvili & Garret 2005). This is quite different in comparison to bacterial viruses, which are almost exclusively using the head-tail morphology. The genomes of crenarchaeal viruses are similarly unique. The genes of different, sequenced crenarchaeal viruses share very little or no homology with other viruses or cellular organisms, suggesting that they are ancient and have emerged independently (Ortmann et al. 2006, Prangishvili et al. 2006). Crenarchaeal viruses also have various unique characteristics. SIRV1 is a rudivirus that appears to have a mechanism by which the genome can rapidly undergo major variation in response to the particular type of host it is currently infecting (Peng et al. 2004). ATV is an *Acidianus* infecting virus that was shown to be the first virus ever to go major development outside of the host as ATV builds a tail structure in extracellular environment (Prangishvili et al. 2006). Furthermore, ATV was exceptional for an archaeal virus because it has multiple transposable elements within its genome.

The unusual diversity of crenarchaeal viruses has not yet been explained. However, Snyder and colleagues studied the mechanism of maintenance of the virus diversity in Yellowstone over two years and came to the conclusion that crenarchaeal virus diversity persists because viruses are readily exhanged between different, geographically isolated hot springs (Synder et al. 2007). In this thesis the potential reasons behind crenarchaeal diversity is discussed (VIII).

1.4.6 Prokaryal viruses and host evolution

Viruses can influence the evolution of cells in various ways. They can, for example, induce selection pressure to their hosts and cause natural selection to favor certain cellular traits. Or they can introduce novel genes to the host genomes and thus provide the cells with novel functions. Bacteriophages are very abundant and in some views even dominate the evolution of their hosts (Hendrix et al. 1999). Any given and otherwise beneficial trait might not become common among bacterial populations, if it happens to make the bacterium even slightly more susceptible to bacteriophage infections. It has been shown that, in microcosm experiments bacteriophages and bacteria rapidly coevolve (Buckling & Rainey 2002a). Viruses can cause sympatric diversification of their hosts if there are multiple strategies by which virus-resistance can be acquired (Buckling & Rainey 2002b). Viruses can also accelerate the rate by which separated populations evolve (Buckling & Rainey 2002b). However, some have suggested that viruses have even more profound effects in natural environments (Day 2004). This is due to (usually) lower resources in nature, where the environment is also discontinuous.

Viruses can also directly alter the life strategies of their hosts. Certain bacterial viruses can, for example, convert sporulation-defective bacteria into sporulating bacteria (Bramucci et al. 1977, Perlak et al. 1979, Silver-Mysliwiec & Bramucci 1990). Keggins et al. in 1978, demonstrated several phages, belonging to different virus groups, to be able to make *Bacillus pumilus* to sporulate. The structure of a repressor – anti-repressor complex responsible for sporulation, suggests that sporulation and prophage induction (i.e. virus formation from a lysogenized cell) are evolutionarily related (Lewis et al. 1998). Nevertheless, viruses can crucially contribute to the host survival. The endospores, for example, are highly resistant to environmental conditions and thus sporulation can help bacteria to survive various types of catastrophes.

Evolution of many bacterial genomes appears to be greatly influenced by phages. Viruses can be seen as effective genome editing agents as they can introduce a variety of novel genes into the host in a single evolutionary event (Witzany 2006). Many bacterial genomes contain prophage or phage remnants. These can, for example, encode virulence factors that render avirulent bacteria into disease causing agents (Brussow et al. 2004). Indeed, prophages can make up to 10-20% of the genetic material in a bacterial chromosome and several genes belonging to these otherwise defective prophages may retain their functionality (Casjens 2003). Viruses also mediate the horizontal transfer between organisms. For example, Kidambi and colleagues showed in 1994 that Pseudomonas phages have horizontally transferred genes between bacteria. Viruses also serve as agents of gene movement between distantly related cells (Canchaya et al. 2003).

In this thesis the effect of bacteriophages on host evolution is studied in two different experimental settings (IV, V). Furthermore, the potential role of viruses in the evolution of cells is discussed in most of the original publications.

1.5 Origin of cells and viruses

All things that most people are willing to denote to be living organisms (without an exhaustive discussion on what life is and what can be considered as living beings) are formed of cells. However, even the simplest, independently surviving and propagating cell has been argued to require a minimum genome of something between two hundred to around four hundred genes. These numbers have been derived from genome comparison studies and mutation/knockout experiments. When some of the first bacterial genomes came available, 256 orthologous genes that were shared by Gram-negative *Haemophilus influenzae* and Gram-positive *M. genitalium* was suggested to be close to the minimal set of genes (Mushegian & Koonin 1996). Later in 2004 when genomes of several free-living and endosymbiotic bacteria became available, Gil et al proposed 206 genes to form the core of a minimal bacterium. In 2009 Lapierre and Gogarten attempted to determine the size of the 'pan-genome' (i.e. the set of all genes present in the group of organisms) of bacteria by comparing 573 genomes. They came up with a number 250 for the amount of genes in the pan-genome. Disruption and knockout studies indicated 279 genes of *Bacillus subtilis* to be essential (Kobayashi et al. 2003, Westers et al. 2003). *M. genitalium* genome consists of 482 genes of which 382 appear to be necessary for the free survival of the bacterium (Glass et al. 2005). Given all of these numbers it is clear that even the simplest functional genome could not have emerged just by pure chance within the lifetime of the universe. Thus natural selection must have been driving the evolution of life even before the emergence of the first independent cells.

Studies on protein sequence differences, i.e. molecular clocks (see Feng et al. 1997), suggest bacterial and archaeal domains to have separated from each other about four billion years ago (Battistuzzi et al. 2004). Eukarya, however, emerged about billion years later than Bacteria (Hedges et al. 2001, Hedges et al. 2004). Nevertheless, about 3.5 billion years ago most of the principal biochemical pathways that sustain the modern biosphere were already globally distributed on Earth (Nisbet & Sleep 2001). Eukaryal genes are generally more closely related to Archaea than to Bacteria, despite of the presence of bacterial endosymbiont (mitochondria) in almost all eukaryal cells (Brown & Doolittle 1997). This suggests that Eukarya evolved from an ancestor, which was related to Archaea.

Some studies indicate that primitive translational factors were present in the LUCA (Kyrpides & Woese 1998). Mechanosensitive ion channels of Archaea, Bacteria and Eukarya are also apparently evolutionary related, indicating that primitive cells probably used such enzymes for manipulating ion-flux in and out of the cell (Kloda & Martinac 2002). Moreover, the last common ancestor of cells had proteins for SecY subunit of the protein-conducting channel, the signal recognition particles and particle receptors, signal peptidase and proton ATPase (Jekely 2006). In order to explain these

findings, Jekely argued that a hydrophobic layer, possibly a lipid membrane, surrounded the common cellular ancestor. However, for example, modern bacterial membrane lipids are synthesized within the cell and then transferred to the membrane (Andersson et al. 1965, Doerrler 2006), therefore it is likely that the synthesized lipids replaced the early, perhaps abiotically formed, membranes at some point.

Archaeal cells have unique lipids that are composed of isoprenoid side-chains stereospecifically ether linked to sn-glycerol-1-phosphate. Boucher et al in 2004 conveyed a large-scale database survey to study the origin of the archaeal machinery for producing lipids. They suggested that the isoprenoid biosynthesis apparatus evolved through a combination of processes, including co-option of ancestral enzymes, modification of enzymatic activities, gene displacement and integration of components from proto-eukaryal and bacterial genomes. Altogether, given the common features in the three domains of life, it appears that LUCA was a moderately complex organism (or more likely a community of organisms), but perhaps not yet an independently surviving cell (Koonin et al. 2006).

Emergence of cell walls has been argued to be an important step in the evolution of primordial cells because murein allowed the cells to maintain higher osmotic pressure inside the cell (Koch 2006, Koch 2003). Koch argued in 1998 that the origin of murein might have been the point when the bacterial cells truly diversified into a stable domain of their own. Therefore Gram-positive rods might have been the first bacterial cells (Koch 2003). Somewhat similarly Zorzopulous suggested in 2003 that all the three domains of life are independent isolates of the primordial proto-cell community. Zorzopolous argued that these isolations could have occurred due to the emergence of murein in bacteria, glycoproteic cell wall in Archaea and nuclear memrane in Eukarya. Forterre suggested in 2006 that perhaps it was three different DNA viruses that established the three domains of cellular life by providing a mechanism for replicating the three primordial cellular RNA genomes. DNA polymerases of cells might indeed be of viral origin (Koonin 2006).

Origin of Eukarya must have been somewhat different from the origin of prokaryotes, because eukaryal cells compose of intracellular symbionts (i.e. mitochondria) and a highly complex endomembrane system. In 2007 Poole and Penny evaluated some of the presented hypotheses for explaining the origin of eukaryotes and especially the origin of mitochondria in eukaryal cells. The first model they surveyed was a scenario in which an archaeon fused with a bacterium to form a genomic fusion of mother cells. In the second alternative some undefined cell begun to host an archaeon and a bacterium, and eventually the former prokaryote turned into the nucleus and latter into mitochondria. The third evaluated hypothesis stated an archaeon to have engulfed a bacterium and then this archaeon evolved into a eukaryote. The fourth model depicted a proto-eukaryote to have engulfed a bacterium. In the conclusion Poole and Penny came to favor the last model and emphasized that the ancestor of Eukarya probably had an ability to engulf other cells. Rivera and Lake studied

the complete genomes of various organisms and suggested eukaryotes to be a result of fusion of an archaeon and either a proteobacterium or a deeply branching clade of bacteria, which included both the Cyanobacteria and the Protobacteria (Rivera & Lake 2004). Some have suggested that the nucleus is of viral origin. Takemura, for one, proposed that poxvirus-like virus could have served as a proto-nucleus for a primitive eukaryote (Takemura 2001). Vesteg et al. suggested in 2006 that the endomembranes of eukaryotes might have evolved from prokaryotic inner membrane and the cell membrane of eukaryotes was originally the outer membrane of a Gram-negative bacterial cell.

Viruses have (most probably) originated several times independently in the history of life (Bamford 2003), and thus emergence of a single virus-type might differ from the others. There have been three distinct, mainstream models for explaining the origin of viruses. The three models are the reduction model, escaped genes model and virus first model. However, the true *de novo* origin of viruses must not be mistaken for the emergence of new disease causing viruses such as Human immunodeficiency virus (HIV) or Severe Acute Respiratory Syndrome (SARS) virus (Tailor et al. 2003, Haagmans et al. 2009, Graham & Baric 2010). HIV, for example, is closely related to other lentiviruses and it is only a slightly modified version of the primate infecting Simian immunodeficiency virus (SIV) (Simon et al. 1992, Narayan et al. 1995, Heeney et al. 2006). Moreover, the replication of HIV requires a reverse transcriptase, an enzyme that appears to be of truly ancient origin (Koonin et al. 2006). The true origin of essential virus genes is thus, arguably, a different issue from the origin of new virus variations or virus "species" (Krupovic & Bamford 2007, Forterre 2009, Krupovic et al. 2009).

The reduction model hypothesis suggested that viruses could be degenerated cells that adopted a parasitic life strategy and the virus-like replication mechanism (i.e. virion assembly). There are indeed many intracellular parasites that are cells themselves. Claverie and colleagues argued in 2006 that giant Mimivirus with its 911 genes (some of which are usually found only from cellular organisms) resembles an intermediate between a virus and a prokaryotic intracellular parasite. However, this view has been criticized as Mimivirus apparently is still a virus (just a complicated one) and the prokaryotic parasites are still clearly related to cells and have no virus-like features (expect the parasitic life-strategy) (see e.g. Desjardins et al. 2005, Villarreal & Witzany 2010). Instead, a great number of Mimivirus genes have been acquired horizontally from amoeba and bacteria (Moreira and Brochier-Armanet 2008), and thus mimivirus and other huge viruses have evolved from smaller viruses into large ones and not vise versa (Villarreal & Witzany 2010). Generally, reduction model appears to be falsified by current evidence since viruses have many conserved genes that are prevalent in viruses but cannot be found from cellular organisms (Koonin et al. 2006).

The escaped gene hypothesis suggests that perhaps some extra-chromosomal elements acquired a capsid-encoding gene and evolved into a

virus (Hendrix et al. 2000). The first capsid gene perhaps emerged from some pre-existing gene through mutations, which allowed the gene product assemble into an icosahedron, for example. These capsids then might have enclosed certain genes inside and occasionally become transferred from one cell to another. Hendrix and colleagues suggested that these escaped genes eventually evolved into true viruses. Krupovic and colleagues demonstrated that Geminiviruses appear to have originated from a phytoplasmal plasmid by acquiring a capsid gene that is related to Satellite tobacco necrosis virus. Escaped gene hypothesis may be true in some sense. The escaped genes could have provided the early organisms a protocol for sharing genetic innovations between cells (Hendrix et al. 2000). When escaped genes hypothesis is formed in this way, it is possible that the escaped genes and the virus-first models overlap (see below). However, there probably were no true cells from which the genes could have escaped at this early evolutionary period.

The virus-first model suggests that viruses emerged simultaneously or even before the first cells. Parasitic virus-like agents might have played important role in the evolutionary community of the primordial replicators (Koonin et al. 2006). The virus-first model is supported with multiple observations. Major capsid proteins have been argued to serve as good targets for inferring the evolutionary history of viruses (Tidona et al. 1998). Benson et al suggested in 1999 that the coat proteins of PRD1 and Adenovirus might be evolutionarily related because both viruses share the double beta-barrel (or the so-called jellyroll) fold in their major capsid proteins. Moreover, the spike protein P5 of PRD1 appears to be related to that of Adenovirus (Merckel et al. 2005). In 2005 Khayat and colleagues showed that also a *Crenarchaea* infecting icosahedral, membrane containing Sulfolobus Turreted Icosahedral Virus (STIV) encodes a coat protein with a double-jellyroll fold. Krupovic and Bamford extended this lineage to *Euryarchaeota* by demonstrating two proviruses in euryarchaeal genomes to be similar to STIV (Krupovic & Bamford 2008). In 2007 Akita and colleagues discovered hyperthermophilic archaeon *Pyrococcus furiosus* to produce virus-like particles that resemble structurally Herpesviruses and tailed bacteriophages. Since all these viruses appear to infect very divergent host organisms, it is most likely that the ancestor of these viruses existed before the host diverged from each other. Viruses might have emerged very early in the history of life, probably already before the last universal common cellular ancestor. Koonin and colleagues developed the so-called "Ancient virus world" hypothesis (Koonin et al. 2006). They suggested that viruses must be of early origin and the role of viruses in the early evolution of life was very important. In order to support the early origin of viruses they argued that several genes coding for viral replication, morphogenesis and capsid formation are shared by several viruses but are missing from cellular life forms. In their concept, the primary lineages of viruses and related selfish agents emerged from the primordial pool of primitive genetic elements. The RNA viruses were the first to evolve, and retroid elements and eventually DNA viruses followed them. The viruses emerged before the modern cells and thus

the early viruses allowed extensive gene mixing within the emerging living system. The virus-genes that formed during the first steps of evolution were suggested to have direct and uninterrupted descendants among modern viruses.

In this thesis the origin of viruses and the role of viruses in the origin of cells are discussed (I, VI, VII and VIII). Next I will review briefly some of the models and experiments concerning the early evolution of life.

1.6 Early evolution of life

Life emerged about four billion years ago (Battistuzzi et al. 2004). The very first life forms must have been something relatively simple. Competition, selection and evolution in general eventually increased the complexity of the primitive life.

Alkaline, warm hydrothermal vents with bubble-like micro-compartments have been suggested to serve as an ideal alternative to the earlier "primordial soup" hypothesis for the location of origin of life (Russell et al. 1994). Compartmentalization has been argued to be essential for early life because it helped the emerging life to maintain the integrity of molecular systems (associated with metabolism) and allowed different systems to vary in their compositions (Monnard & Deamer 2002). In 2005 Koonin and Martin refined the previously presented idea that life emerged on submarine hydrothermal vents. Their scenario suggested that life originated in a warm geothermally heated vent within a matrix of contiguous iron-sulfide compartments where primitive virus-like RNA molecules, perhaps encoding some proteins, evolved. The first "cells" were not free-living independent organisms but instead a compartment-dependent populations of replicators and other agents. Martin and Russell have suggested that the basic biochemistry was the first to emerge in these alkaline vents and only later the genetic replicators appeared to the scene (Martin & Russell 2007). Nevertheless, some sort of biological replicators had to emerge eventually and thus kick-start the Darwinian evolution of life. Miller and Bada had previously criticized the hydrothermal vent hypothesis for being too optimistic, as the high temperatures in vents would decompose any organic molecules and especially any replicators that were formed of RNA-polymers (Miller & Bada 1988). However, there are several types of thermal vents and the first discovered 400 °C "black smokers" are not the only ones. Indeed, the temperature of the "lost-city" type of an alkaline vent is only around 40 – 90 °C (Martin & Russell 2007). Nevertheless, there is a thermal gradient from the hot interior of the vent to the ice-cold seawater, and thus at some radius from the vent core the temperature would be suitable for most biochemical reactions (Braun & Libchaber 2004). Others have pointed out that ocean floor vents might have been relatively safe places for life to evolve during the heavy meteorite and comet bombardment of early Earth (Nisbet & Sleep

2001). The plausibility of some aspects of the hypothetical hydrothermal vent origin of life has been experimentally studied. It was noticed, for example, that nucleotides accumulated rapidly into the simulated hydrothermal vent pores, suggesting that the raw material for nucleotide polymerization could concentrate within such environments (Baaske et al. 2007). Moreover, thermal gradients within the vents could drive molecular evolution as nucleotide polymers are continuously cycled between hot and cold regions of the chamber (Braun & Libchaber 2004). However, environmental flux, in which nucleotide monomers are constantly changing, appeared also to cause the nucleotide polymers evolve more slowly towards the expected equilibrium (Epstein & Eigen 1979).

It is evident that life begun to use nucleotides at some point in the early evolution of life. The RNA-world hypothesis (see e.g. Orgel 2004, Dworkin et al. 2003) states that primordial life used RNA for genetic storage and for enzymatic functions. In some simulation experiments RNA replicases become prevalent in a nucleotide pool (Ma et al. 2007a). Ma and colleagues also argued that the first ribozymes to be favored by natural selection would have been nucleotide synthases (Ma et al. 2007b) and the first replicases would be template-dependent RNA ligases (Ma et al. 2010). RNA-molecules have also been shown to bind membranes (Janas et al. 2006) and form e.g. tryptophan transporters (Janas et al. 2004). The early membrane bound life forms may have benefitted from the ability of RNA to change the permeability and other characteristics of lipid membranes (Khvorova et al. 1999). However, Laiterä and Lehto proposed in 2009 that early replicators utilized membrane-surrounded vesicles only after the emergence of genetically encoded proteins, and not during the early stages of the RNA-world. These early proteins could selectively direct specific molecules into vesicles. This, in turn, would protect the replicators against competition and parasites. Some viruses still utilize such vesicles for isolating the virus replication from the biochemical processes of the host. Wolf and Koonin suggested a scenario for explaining the origin of translation machinery (Wolf & Koonin 2007). In their model the primary cause for the emergence of translation is the ability of amino acids and simple peptides to enhance ribozyme activity.

Lipids and membranes could be synthesized in prebiotically plausible conditions and thus it is possible that at least some of the early life forms became enclosed within hydrophopic layers (Hargreaves et al 1977). Monnard and Deamer established conditions in which monomer substrates, such as ATP, could diffuse into simple lipid vesicles (Monnard & Deamer 2001). Different types of lipid molecules appear to have different advantages in forming useful vesicles from the point of view of an emerging life (Furuuchi et al. 2005). Interestingly, Chen and colleagues showed that the vesicles, which are able to collect most molecules (such as nucleotides) within themselves, are bound to evolve in size as the osmotic pressure on the vesicle membrane drives the uptake of additional membrane components from the environment (Chen et al. 2004). This suggests that any property of a vesicle that allows more nucleotides

to accumulate within the vesicle is favored over the other vesicles in Darwinian sense. In a community of prebiotic vesicles, group selection might also play an important role in the evolution of primordial replicators (Fontanari et al. 2006). Term *group selection* refers to a case where the phenotype of a certain gene benefits the whole group of organisms (or replicators) rather than the gene itself. While group selection does not operate (significantly) among the evolution of modern organisms, it might sill play a relevant part within the early life (Szathmary & Demeter 1987).

Compartmentalization and spatial distribution of replicator communities apparently can have positive effects on the evolution of such systems, as parasites are less likely to invade the whole system (Hogeweg & Takeuchi 2003). However, some have suggested that in optimal systems the replicators should be immobilized on surfaces of the compartments rather than allowing them to freely diffuse in liquid cultures (Scheuring et al. 2003). It has also been suggested that viruses might have played an important role in the evolution of early life and the emergence of cellular domains (Forterre 2005). Villarreal and Witzany argued in 2010 that viruses are essential agents in the stem of life and we need to include them into the scenarios of early life.

The role of virus-like genes in the primordial ribozyme-community was discussed (VII) and studied in this thesis (VI).

2 AIMS OF THE STUDY

The overall purpose of the study was to investigate some potential ways by which viruses influence the evolution of (early) life and to better comprehend the underlying reasons in the apparent association of the emergence of viruses and the evolutionary processes occurring on primordial Earth.

The more exact aims were:

1. To study the genetic relationship of the structurally similar viruses P23-77 and SH1
2. To determine whether these viruses are related to other icosahedral, inner-membrane containing viruses
3. To study the effect of bacteriophage selection on the evolution of their hosts in different experimental settings
4. To study the potential benefits of virus-like agents in simulated conditions representing the hypothetical evolutionary community of the primordial life

3 MATERIALS AND METHODS

The complete materials and methods are described in the original publications. Here the materials and the computational methods are only briefly reviewed. The bacterial strains, plasmids and viruses that were used in the thesis are listed in Table 1.

TABLE 1. Bacteria, plasmids and viruses used in the thesis

Bacteria	**Used in study**
Escherichia coli K-12	I, IV, IX
Salmonella enterica serovar Typhimurium	IV
Serratia marcescens ATCC 13880	V
Meiothermus ruber DSM 1279	II
Meiothermus silvanus DSM 9946	II
Thermus thermophilus ATCC 33923	I
Bacillus thuringiensis	III, IX
Bacillus anthracis	III, IX

Plasmids	**Type**	
pLM2	IncP, Conjugative	IV
RP4	IncP, Conjugative	IV
RN3	IncN, Conjugative	IV
pSU18	Cloning	I
pMG60	Cloning	IX

Viruses	**Virus family**	
PRD1	*Tectiviridae*	IV, IX
AP50	*Tectiviridae*	III, IX
Bam35	*Tectiviridae*	IX
PPV	*Podoviridae*	V
P23-77	Unassigned	I

All sequencing reactions (I, II, III, V) were performed using the BigDyeTM Terminator v3.1 Sequencing Kit on an ABI PrismTM 3130 automated sequencer (both from Applied Biosystems). Sequence assembly and all sequence analyses were done using Vector NTI Advance 10 (I, III) or 11 (II, V) (Invitrogen Inc.). Moreover, the following computational software and resources were used in the thesis. BLAST and PSI-BLAST (Altschul et al. 1997) were used for searching similarities with putative proteins from global sequence databases (I, II, III, V, IX). SMART (Letunic et al. 2006) was used for domain recognition in putative proteins (I, II, III, V). PHYRE (Bennett-Lovsey et al. 2008) was used for predicting structural similarities in putative proteins (I, II). PrimordialEvo (VI) was used for simulating the evolutionary dynamics within a community of ribozyme-like agents. MEGA 4.1 (Tamura et al. 2007) was used for inferring the evolutionary histories of virus proteins (I, II). Bodil (Lehtonen et al. 2004) was used to visualize and compare Protein Data Bank deposited structures (IX). SPSS was used for statistical analyses (IV, V).

4 REVIEW OF THE RESULTS

4.1 Genome sequence and characteristics of the *Thermus* phage P23-77 (I)

P23-77 is an icosahedral, inner membrane containing bacteriophage infecting *Thermus thermophilus* (which was originally designated as *Thermus flavus*). The genome of P23-77 is 17036 bp circular double-stranded DNA. The genome of P23-77 contains 37 putative genes (Table 2). Five genes encode proteins that were determined to be associated with the internal membrane of the virion. Three genes encode proteins that are only part of the protein capsid. Among the putative genes there are genes for a conserved ATPase, a DNA replication initiation protein similar to some *Thermus* plasmids (De Grado et al. 1998, Ruan & Xu 2007), a transglycosylace, a peptidase and an amidase. However, significant portion of the genes show no similarity to any other previously sequenced genetic entities (excluding a closely related virus IN93 and the integrated elements in *Meiothermus* genomes, see below). The lipid pattern of P23-77 virion was noted to differ from the host membrane. P23-77 appears to be evolutionarily related to *Thermus* phage IN93, archaeal plasmid pHH205 (Ye et al. 2003), integrated genetic element in *Haloarcula* (IHP) and the *Haloarcula* virus SH1. The ATPase of P23-77 is similar to those of PRD1-adenovirus lineage. The structural prediction program PHYRE suggested the large major capsid protein of P23-77 to be structurally similar with a double-beta barrel capsid protein of Sulfolobus Turreted Icosahedral Virus (STIV).

TABLE 2 Annotation of the genome of *Thermus* phage P23-77

ORF P23-77	**Residues** (position)	Determined proteins (mass)	Predicted Function (BLAST)
1	**425** (241 - 1515)		DNA replication protein
2	**125** (1515 - 1889)		
3	**255** (1889 - 2653)		Phosphoadenosine phosphosulfate reductase
4	**129** (2653 - 3039)		
5	**97** (3070 - 3360)		
Gene 6	**187** (3456 - 4016)	22 kDa	VP6
7	**131** (4068 - 4460)		
8	**173** (4378 - 4896)		
9	**203** (4829 - 5437)		Endolysin
10	**178** (5443 - 5976)		Amidase endolysin
Gene 11	**187** (5996 - 6556)	26/24.5 kDa	VP11
12	**64** (6556 - 6747)		
13	**224** (6740 - 7411)		ATPase
14	**66** (7414 - 7611)		
Gene 15	**138** (7556 - 7969)	15 kDa	VP15
Gene 16	**173** (7983 - 8501)	20 kDa	Small major capsid protein (VP16)
Gene 17	**291** (8514 - 9386)	35 kDa	Large major capsid protein (VP17)
18	**116** (9407 - 9754)		
Gene 19	**87** (9761 - 10021)	8.5 kDa	VP19
Gene 20	**227** (10021 - 10701)	30 kDa	VP20
21	**72** (10704 - 10919)		
Gene 22	**92** (10912 - 11187)	14 kDa	VP22
Gene 23	**75** (11199 - 11423)	12 kDa	VP23
24	**140** (11423 - 11842)		
25	**41** (11842 - 11964)		
26	**62** (11960 - 12145)		
27	**150** (12127 - 12576)		
28	**341** (12651 - 13673)		
Gene 29	**369** (13686 - 14792)	40 kDa	Lysozyme (VP29)
30	**88** (14795 - 15058)		
31	**233** (15039 - 15737)		Transglycosylase
32	**72** (15997 - 16212)		
33	**62** (16208 - 16393)		
34	**35** (16393 - 16497)		
35	**90** (16503 - 16772)		
36	**145** (16772 - 170)		
37	**78** (8 - 241)		

4.2 Genomic sequences of phage IN93 and the integrated virus-like elements in *Thermaceae* and *Halobactericeae* (I, II)

Several of P23-77 genes were noticed to be similar to a GenBank deposited virus sequence of the *Thermus* phage IN93. The genome of IN93 is 19604 bp long and

it contains 39 putative genes. 25 of the IN93 genes are clearly homologous with P23-77, whereas the rest show no or very little similarity. However, interestingly IN93 contains a set of genes that are encoded to the opposite direction in respect to the other genes. These genes are absent in P23-77 genome. The set includes genes for a bacteriophage integrase, a restriction endonuclease and a prophage repressor. Such functions are often required in the process of host chromosome integration or in the maintenance of the prophage stage in bacteriophages. Generally the corresponding proteins of IN93 and P23-77 are 80% identical.

Searching of the GenBank database with P23-77 sequence as a reference revealed similar genes in the genomes of *Meiothermus silvanus* and *Meiothermus ruber*. Eventually five genome-integrated elements containing the genes for the major capsid proteins and the putative genome packaging ATPase of P23-77 were discovered. These elements were designated as MeioSilP1, MeioRubP1, IHP, HaloMukP1 and HaloMukP2 for the elements in the genomes of *Meiothermus silvanus*, *Meiothermus ruber*, *Haloarcula marismortui*, *Halobacterium mukohatei* and *Halobacterium mukohatei*, respectively. Elements in *Meiothermus* genomes are located next to the transfer RNA (tRNA) genes, which are common sites for bacteriophage integration. Also IN93 sequence has some nucleotides that are similar with tRNA genes. The general characteristics of all of these elements and related viruses are listed in Table 3.

TABLE 3 Members of the lineage of P23-77-like (single beta-barrel) viruses

Element	Type	Genome size (bp)	Genes	Genome topology	Host domain	Host organism
P23-77	Virus	17036	37	Circular	Bacteria	*Thermus thermophilus*
IN93	Virus	19604	39	Circular	Bacteria	*Thermus aquaticus*
MeioSilP1	Integrated provirus	~21110	45	n/a	Bacteria	*Meiothermus silvanus*
MeioRupP1	Integrated provirus	~16019	32	n/a	Bacteria	*Meiothermus ruber*
SH1	Virus	30898	56	Linear	Archaea	*Haloarcula hispanica*
pHH205	Plasmid	16341	32	Circular	Archaea	*Halobacterium salinarum*
IHP	Integrated provirus	~19000	34	n/a	Archaea	*Haloarcula marismortui*
HaloMukP1	Integrated provirus	~16000	26	n/a	Archaea	*Halomicrobium mukohataei*
HaloMukP2	Integrated provirus	~20000	30	n/a	Archaea	*Halomicrobium mukohataei*

4.3 P23-77-like viruses and virus-like elements form a globally distributed viral lineage spanning the domains of Bacteria and Archaea (II)

Three homologous genes are present in the genomes of *Thermus* phages P23-77 and IN93, *Haloarcula* virus SH1, *Halobacterium* plasmid pHH205, *Meiothermus* elements MeioRubP1 and MeioSilP1, *Haloarcula* element IHP and *Halobacterium* elements HaloMukP1 and HaloMukP2. Two of the genes encode major capsid proteins and one encodes a putative genome packaging ATPase. P23-77 and SH1 are also structurally similar as both viruses decorate their capsomers with tower-like structures and contain an inner membrane beneath the icosahedral capsid (Jaatinen et al. 2008, Jäälinoja et al. 2008). Moreover P23-77 and SH1 use triangulation number 28 to arrange the major capsid proteins to yield an icosahedron. The viruses and the virus-related elements are distributed around the globe (Fig. 8). Phylogenetically the viruses and elements in Archaea form a distinct branch from the viruses and elements in Bacteria (Fig. 9).

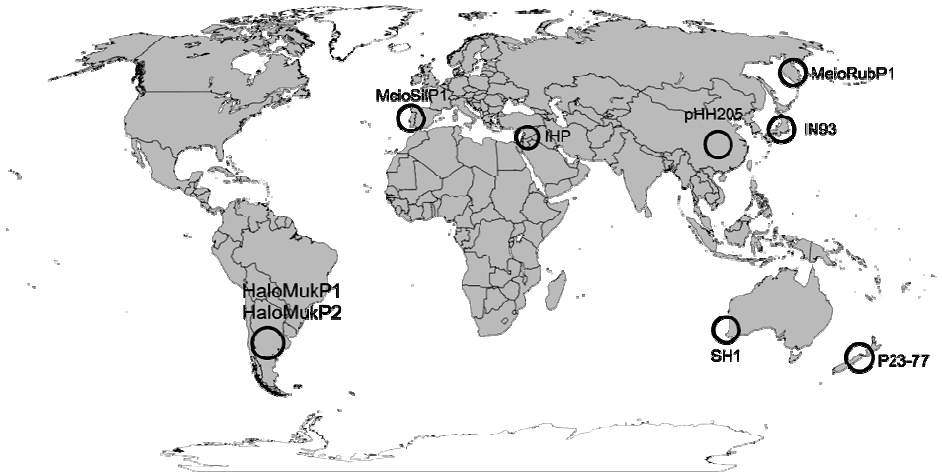

FIGURE 8 Geographical distribution of the locations of the viral and prokaryotic isolates that host the genetic elements.

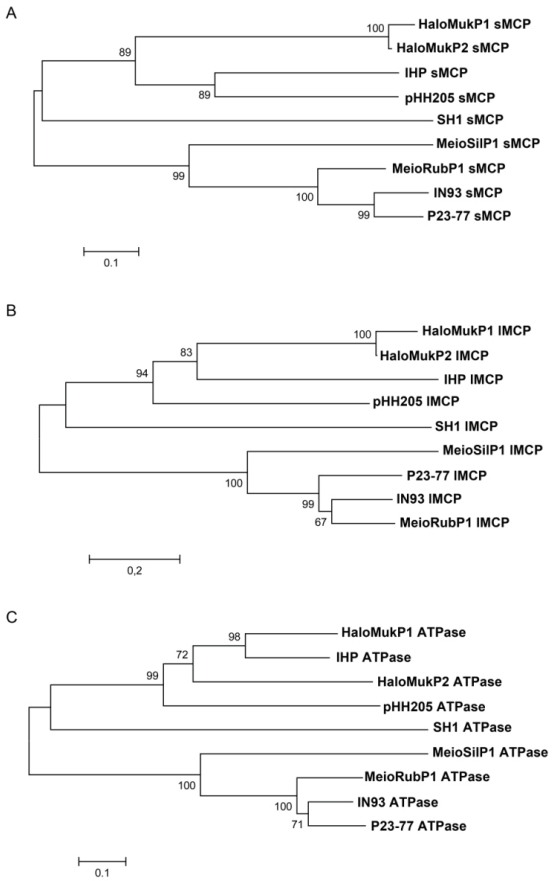

FIGURE 9 Minimum evolution tree of (A) the small major capsid protein sequences, (B) the large major capsid protein sequences and (C) the ATPase sequences of the members of the single beta-barrel lineage. The evolutionary histories were inferred using the Minimum Evolution (ME) method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method. The tree was searched using the Close-Neighbor-Interchange (CNI) algorithm. The initial tree was generated with the Neighbor-joining algorithm. All positions containing gaps and missing data were eliminated from the dataset.

4.4 Genome sequence of *Bacillus anthracis* phage AP50 (III)

AP50 is an icosahedral, inner membrane containing virus infecting *B. anthracis* that belongs to the family *Tectiviridae* (Ackermann et al. 1978). The topology of double stranded DNA genome is linear and it was determined to be 14398 bp long. In 1974 AP50 was incorrectly reported to be a RNA virus (Nagy et al. 1976). In the study III the AP50 genome was confirmed to be composed of

deoxyribonucleotides. However, Nagy et al. had also corrected their error already in 1977. AP50 genome contains 31 putative genes (Fig. 7). Majority of the genes are related to the previously sequenced Gram-positive bacteria infecting tectiviruses Bam35, Gil01, Gil16 and pBClin15. However, two distinct regions are very different in these virus genomes. The first is located after ORF 7 and the second is located at the end of the genome after ORF 27. The lysis proteins of Gil16 and AP50 showed variability in their C-termini regions, suggesting evolution in the protein's cell-wall binding domain (Kuroda et al. 1992). First case of non-orthologous gene replacement among tectiviruses was identified when the lysins of Gil16 and AP50 were shown to be related to each other but not to those of Bam35 and pBClin15. Moreover, for the first time a gene for a MerR-type regulator was identified from a tectivirus genome as the ORF1 of AP50 is similar to several MerR-regulators. However, these regulators can have multiple nucleotide recognition sequences (Parkhill et al. 1998, Hobman 2007) and thus the binding site of the protein could not be determined. ORF1 of AP50 is similar to the first ORFs of Bam35, Gil16 and pBClin15, suggesting a conserved regulatory role for this protein in these viruses. Single nucleotide mutations at specific positions in either end of the AP50 genome produced clear-plaque variant of AP50. Potentially these mutations altered the regulator binding sites and made the phage unable to repress the structural and lysin genes. The *B. anthracis* AP50-lysogens formed wrinkled and flat colonies whereas the AP50-free cells produced smooth and round colonies.

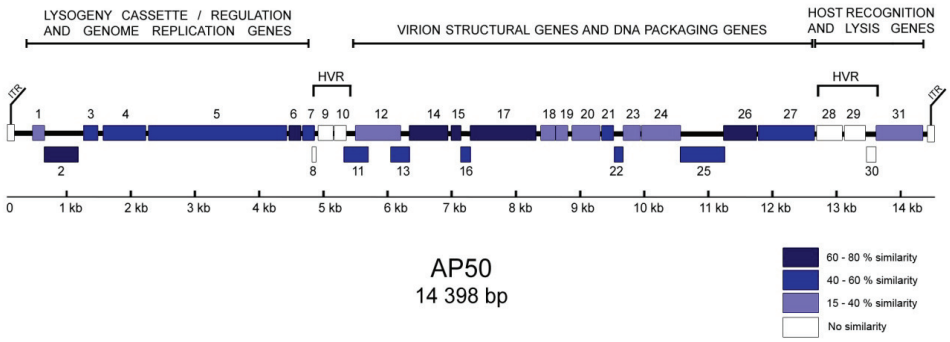

FIGURE 7 The genome of AP50. The putative genes are marked with boxes. The color scheme of the box indicates the mean similarity on amino acid level in comparison to the corresponding proteins in Bam35, Gil16 and pBClin15. HVR, highly variable region; ITR, inverted terminal repeat.

4.5 Plasmid-dependent bacteriophage selects plasmid-free cells in bacterial populations (IV)

PRD1 is a bacteriophage, which exploits the mating pair complex encoded by certain conjugative plasmids (IncP, IncW and IncN) as a receptor. These plasmids encode multiple antibiotic resistances. We tested the effect of PRD1 selection on plasmid-borne antibiotic resistances in bacterial populations during a ten-day serial culture experiment. In the control experiments, where PRD1 was not introduced to cultures, the antibiotic resistances showed no decrease. However, if PRD1 was introduced to the cultures on day one or every day, majority of *Escherichia coli* K-12 and *Salmonella enterica* LT2 Seovar Typhimurium cells harboring an IncP conjugative plasmid RP4 lost their antibiotic resistances. The resistace was even more rapdily lost from *E. coli* cells harboring an IncN plasmid RN3. The evolution of antibiotic resistances in the absence and in presence of PRD1 is shown in Fig. 10. Eighteen antibiotic-susceptible *E. coli* cells and fourteen *S. enterica* cells originally harboring RP4 were confirmed to have lost their plasmid by screening for the absence of three plasmid-specific genes with polymerase chain reaction. The cells that had retained the antibiotic resistance were also PRD1-resistant. Twelve double resistant mutants were tested for their ability to conjugate. None of them could transfer the antibiotic resistance to a recipient cell. Obviously PRD1-selection disturbed the conjugation apparatus encoded by the plasmid.

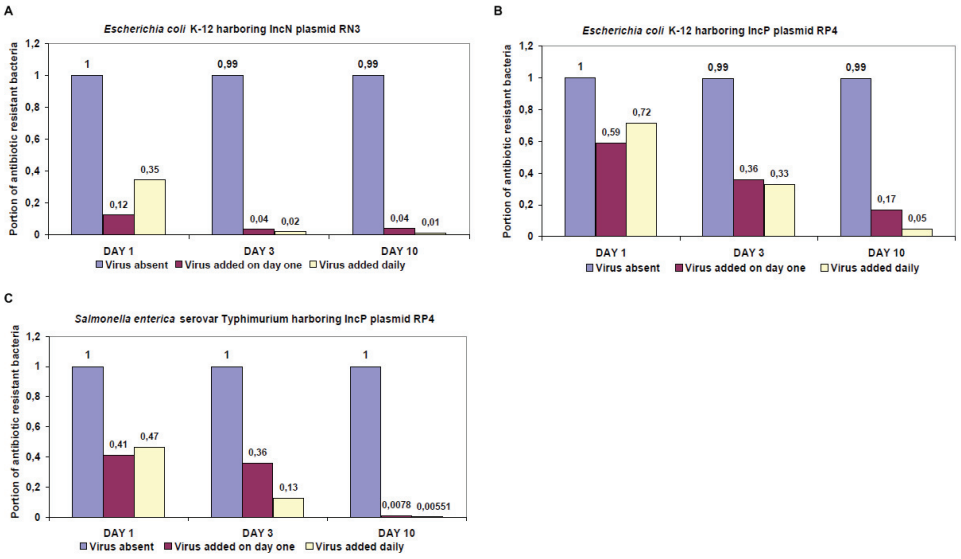

FIGURE 10 Evolution of antibiotic resistance in populations of (A) E. coli harboring IncN plasmid, (B) E. coli harboring IncP plasmid and (C) *S. enterica* harboring IncP plasmid when the plasmid-dependent bacteriophage PRD1 was absent, added on day one or daily.

4.6 Bacteriophage indirectly selects less pathogenic *Serratia marcescens* phenotypes (V)

Serratia marcescens ATCC 13880 was cultivated for a month in a weekly refreshed microcosm setting with and without the presence of a bacteriophage PPV in two temperatures (25 ºC and 37 ºC). Ten bacterial isolates from each microcosm settings were selected and the motility, resource use efficiency, maximum population size and maximum growth rate were measured from the isolates. The bacteriophage selection decreased the bacterial motility and the maximum population sizes. When the isolates were pooled and injected to *Parasemia plantaginis* tiger moth larvae, the highest mortality was observed among those larvae that had been injected with bacteria evolved in 37 ºC in absence of viruses. The viral selection apparently hindered or slowed the evolution of characteristics that improve the virulence of *S. marcescens*.

4.7 Computational system for studying the evolution of a primordial compartment matrix inhabited by ribozyme-like agents (VI)

A rule-based computing system was designed to study evolutionary dynamics of ribozyme-like agents within a compartment matrix. Previously similar systems have been designed for studying bacterial populations (see Gregory et al. 2008, Vlanchos et al. 2006). The system comprises a three dimensional matrix of proto-cells. Within these cells are resources that may be used for ribozyme synthesis, activation of anti-parasites and cell wall production. The simulated ribozyme-like agents may serve as replicators, resource collectors, vesicle inducers, parasites, anti-parasites, cell wall generators and useless genes. Details of the simulator design are described in VI. We observed that the virus-like ribozymes, which are able to induce the vesicle formation and enclosure of genetic material within, are favored in this particular system setup. Systems in which the virus-like genes were allowed to emerge reached the maximum gene count faster than system where vesicle formation was only a rare and random event or common, but random event. However, for these systems to evolve into sufficient complexity, the system required genes that are able to remove replication parasites from the compartments. Indeed, if the system was allowed to evolve replication parasites, then the number of anti-parasite genes was highly increased in the system.

5 DISCUSSION

In this section I focus on discussing things that either has not been addressed in the original publications or that are relevant to the evolution and life from a wide perspective. Exact analysis of the gene contents, evolution of particular genes and changes in related virus genomes are already covered in the primary articles.

5.1 Early emergence of inner-membrane containing icosahedral viruses

The P23-77-like viruses were predicted to have a major capsid protein, which folded into a (single) beta-barrel conformation (I, II, Jäälinoja et al. 2008, Krupovic & Bamford 2009). It was suggested that perhaps P23-77-like viruses form the earliest branch in the lineage of beta-barrel viruses with an icosahedral capsid and an inner membrane. During the writing process of the thesis, a 1.8 Å X-ray structure of the small major capsid protein (VP16) of P23-77 was determined (pers. comm. Jaana Bamford, Ilona Rissanen, Karl Harlos (University of Oxford) and David Stuart (University of Oxford)). Indeed, VP16 appears to form a beta-barrel with an "arm-like" structure that mediates the dimersization of the proteins. The secondary structure of the protein contains seven to eight beta-sheets that arrange into a barrel-like conformation. The "arm" extending from one beta-barrel embraces the barrel of another protein subunit, thus tightly locking the two barrels together. This structure immediately suggests that there is strong tendency for the major capsid proteins to evolve functions, which interlock the proteins into dimers. Therefore evolution towards the PRD1-like double-beta barrel structure (where a single protein encodes two barrels) appears to be a rather logical innovation for solving this structural need. As noted above, PRD1 and P23-77 share also other similarities aside of the somewhat similar major capsid protein structures. Both viruses have an icosahedral capsid with an inner membrane. Moreover, these

viruses have a gene encoding for a conserved ATPase (I, II, Strömsten et al. 2005). Considering all these things together, it is probable or even very likely that the double beta-barrel viruses and the single beta-barrel viruses share a common viral ancestor (and possibly that the double-beta barrel protein evolved from the single-beta barrel by gene duplication). If the PRD1-like viruses and P23-77-like viruses are truly evolutionary related, it would be interesting to determine when this viral ancestor existed. One possible, and perhaps even the most plausible, scenario is to assume that the two viruses diverged from each other already before the last cellular ancestor, as this would help us explain why these viruses can be found infecting so divergent cellular life forms.

Let's discuss this question a bit further by considering another important part of a virus from the same viewpoint: the mechanisms of genome replication. Replication genes of bacterial viruses are known to form modules in the viral genomes (Weigel & Teitz 2006). The tailed phage phi29 shares similar replication system with tectiviruses (Pecenkova & Paces 1999), suggesting that in the past the usual replication strategy of *Podoviridae* might have been replaced in phi29 by a tectiviral replication system. Moreover, the replication strategy of phi29 and tectiviruses could be related to a very unique archaeal bottle shaped virus, ABV (Peng et al. 2007). Among cellular organisms, DNA replication strategies also show great variability (Robinson & Bell 2005). It seems unlikely that several DNA replication mechanisms could have emerged independently within a small community of emerging life forms. Thus the early evolutionary system probably already consisted of relatively separate sub-communities when the DNA replication systems originated (Koonin 2005). Perhaps along with the replication systems evolved multiple structural lineages of viruses. Two of these lineages were the PRD1-like viruses with their double beta-barrel capsid proteins and the P23-77-like viruses with their single beta-barrel capsid proteins (and the capsomer decorating proteins). Both of the lineages share the ATPase gene (that may serve different functions in different viruses). At least in tectiviruses the enzyme is used for DNA packaging, and perhaps it was used for a similar purpose already in the primordial world. Nevertheless, the potential to encode your own energy-machinery must have been huge among these viruses, given that the common ATPase can still be found from archaeal, bacterial and eukaryal viruses that can have very different genomes and assembly mechanisms (Abrescia et al. 2008). Possibly the ancestor of these both lineages emerged in the primordial system. The ancestor probably had an inner membrane derived from the membrane of the host (proto-)cell. However, in the beginning the emerged major capsid proteins must have been quite poor in forming the capsomers and thus further evolution to improve the virus fitness took place. At this point the viruses might have separated into two lineages where the two major capsid proteins evolved different strategies to stabilize capsomer formation. These two strategies have ever since (for four billion years) retained their identity in the genomes of various viruses. This scenario is illustrated in Fig. 11.

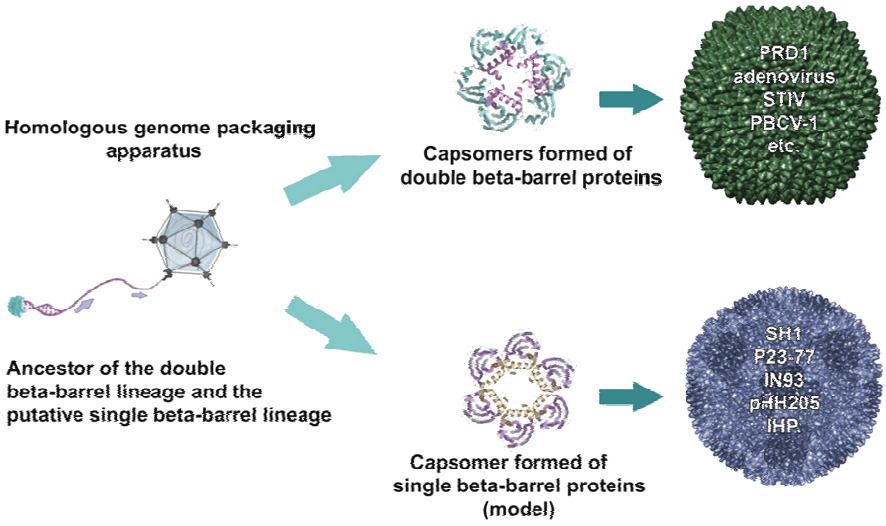

FIGURE 11 A presentation of the model suggesting that the single and double-beta barrel viruses shared a common ancestor.

However, the sizes of the genomes and the capsids of these viruses have been evolving ever since. How and why this evolution could have occurred in some of the single beta-barrel viruses is discussed in the next section. I also make a prediction of the minimum T-number of the primordial ancestor of the beta-barrel viruses, which, in turn, would suggests the number of capsid proteins and genes in the common viral ancestor that existed some four billion years ago.

5.2 A possible (recent) evolution in the capsid architecture among *Thermus* infecting viruses P23-77 and IN93

The size of the virus capsid determines the maximum amount of DNA or RNA it can incorporate, and it is therefore directly linked to the maximum gene encoding potential of viruses. Evolution has provided a virosphere with a great variability in the capsid and genome sizes of icosahedral viruses (Abrescia et al. 2004, Abrescia et al. 2008, Bamford et al. 2002, Iyer et al. 2006, Nandhagopal et al. 2002). DNA-viruses, such as PM2 (infecting *Pseudoalteromonas espejiana*) or PRD1 (infecting a wide range of Gram-negative bacteria), possess genomes between ten and fifteen thousand base pairs (bp) (Männistö et al. 1999), respectively, while the genomic DNA of, for example, Mimivirus (infecting *Acanthamoeba polyphaga*) is over million bps long and is thus indistinguishable to some bacteria by its genetic potential (Raoult et al. 2004). Similarly, PRD1 virion and the Mimivirus capsid (excluding the filaments) are of 80 nm and 400 nm in diameter, respectively. In spite of these differences, they share

homologous major capsid proteins and (putative) genome packaging ATPases (Benson et al. 2004).

Icosahedral viruses have a limited number of ways to assemble the capsid as only certain arrangements of capsid subunits can yield an icosahedron. Triangulation number (T-number), a model developed by Caspar and Klug in 1962, determines the possible icosahedral capsid architectures and almost all of the currently known globular viruses are observed to exploit one of these architectures (excluding a few peculiar examples in which typical capsid hexamers are replaced with pentamers, Grimes et al. 1998, Liddington et al. 1991). The model is based on the fact that each of the twenty facets of an icosahedron should be build of at least three asymmetric protein subunits. Therefore the minimum number of subunit to produce an icosahedron is sixty. However, sixty proteins are likely to yield rather small capsid and thus variations of these asymmetric arrangements of capsomers can be used to increase the number of subunits in the capsid and thus produce larger icosahedrons. Each T-number refers to one of such potential capsomer arrangements and thus T-number also states the amount of capsid subunits required for the assembly of the protein shell (T times 60). Nevertheless, in order to the virus capsid to increase its physical size either the capsid proteins need to expand in diameter or the virus needs to adopt a higher T-number. Both examples are seen in the nature (Benson et al. 2004).

The genome sizes of P23-77 and IN93 differ by 2567 nucleotides. P23-77 uses T=28 to organize the capsid proteins (Jaatinen et al. 2009). T-number of IN93 is not known. If the viruses are assumed to have similar major capsid proteins and their genomes are packed into same density, then the increase of the genome length allows the estimation of T-number by using basic geometry. In other words, in any particular icosahedral virus the T-number is related to the virion surface area and the genome size is relative to the inner volume of the virion. The surface area and volume of natural geometrical shapes always have a specified ratio. Following this line of reasoning, I derived a formula by using the geometry of a sphere (a form these virions closely resemble) to estimate the T-number of a virus by using a related virus as a reference. In other words, the proportions of the areas ($A = 4\pi r^2 = T_{number}$) to the volumes ($V = 4/3\pi r^3 = G_{enome\ length}$) of two viruses were deduced.

The proportions of the inner volumes (V) of the two viruses (1 & 2) are first reduced to the radial (r) components:

$$\frac{V_1}{V_2} = \frac{(\frac{4}{3})\pi {r_1}^3}{(\frac{4}{3})\pi {r_2}^3} = \frac{{r_1}^3}{{r_2}^3}$$

$$\Leftrightarrow$$

$$r_1 = \frac{r_2 \sqrt[3]{V_1}}{\sqrt[3]{V_2}}$$

The proportions of the surface areas (A) of the two viruses are presented below. The r_1 component is then replaced with the above reduced equal:

$$\frac{A_1}{A_2} = \frac{4\pi {r_1}^2}{4\pi {r_2}^2} = \frac{{r_1}^2}{{r_2}^2} = \frac{\frac{\cancel{r_2^2}\sqrt[3]{{V_1}^2}}{\sqrt[3]{{V_2}^2}}}{\cancel{r_2^2}}$$

This gives the basic equation to deal with the inner volumes and the surface areas of the viruses:

$$\frac{A_1}{A_2} = \frac{\sqrt[3]{{V_1}^2}}{\sqrt[3]{{V_2}^2}}$$

$$\Leftrightarrow$$

$$A_1 \frac{\sqrt[3]{{V_2}^2}}{\sqrt[3]{{V_1}^2}} = A_2$$

As stated above, the surface area is related to the T-number of the virus and the inner volume is related to the genome size of the virus. The equation can be modified into the following form:

$$T_1 \frac{\sqrt[3]{{G_2}^2}}{\sqrt[3]{{G_1}^2}} \approx T_2$$

$$\Leftrightarrow$$

(1)

$$T_1 \sqrt[3]{\left(\frac{G_2}{G_1}\right)^2} \approx T_2$$

where T_1 and T_2 are the T-numbers and G_1 and G_2 are the genome lengths of viruses 1 and 2, respectively.

However, it should be noted that the estimations of the formula was tested to be accurate only for relatively similar viruses for which the T-numbers are known (such as PRD1 and Bam35 of the *Tectiviridae* family or Lambda and HK97 of the *Siphoviridae* family). For distant relatives, such as PM2 and PRD1 (members of the *Corticoviridae* and the *Tectiviridae*, respectively), Sulfolobus

turreted icosahedral virus and PRD1 (an unassigned virus and a tectivirus, respectively) or Paramecium Bursaria chlorella virus 1 and Chilo Iridescent virus (members of *Phycodnaviridae* and *Iridoviridae*, respectively), the formula produced more or less incorrect T-numbers (data not shown). This is probably because the packaging densities and the sizes of capsomers are too different in these viruses, and thus the structure of one cannot be used as a precise reference for another.

Nevertheless, we may assume that IN93 capsid subunits are of similar size with P23-77, since these major capsid proteins share about 80% protein sequence identity and the peptide chains are of almost identical lenghts. Moreover, they are much more similar than the major capsid proteins of PRD1 and Bam35, and Lambda and HK97, for which the formula produced correct T-numbers. The formula gives an estimation of ~30.7465 for the T-number of IN93. If the estimation is rounded to the closest allowed T-number, value of 31 is obtained. Interestingly same result comes if PRD1 structure is used as reference (estimation of ~29.98, which also rounds to the closest allowed T-number of 31). This suggests that IN93 might use T=31 for capsid organization.

In order to determine how T=31 arrangement of capsid would differ from T=28 capsid, I built a model of the capsomer arrangement of a T=31 (Fig. 12b) based on the known capsomer arrangement of P23-77 (Fig. 12a, see Jaatinen et al. 2008). P23-77 capsomers have decorating tower-like structures. There are two differently arranged decorations that are located on capsomers in different orientations. In Fig. 12 the decorating structures are marked as spheres within the hexamer, and the two different types are color coded and numbered. The decorating proteins situate similarly in both capsid types, suggesting that no major rearrangements of capsomers are reguired in order to alter the capsid architecture between T=28 and T=31. Interestingly, the T=31 capsid does not require a capsomer situating in the middle of the facet edges (the edge is depicted as a red line in Fig. 12), whereas in T=28 architecture one of the type two capsomers needs to bend in the middle. Moreover, the capsomers of PRD1, for example, follow the edge, thus it is possible that during capsid assembly there is a tendency for T=28 type of a capsid to arrange itself into a, perhaps, more relaxed T=31 conformation. If IN93 actually appears to produce T=31 virion, then the study of the interactions among the capsid proteins of the closely related IN93 and P23-77 could help us understand the differencies that allow viruses to adopt architechtural changes and capsid expansion. Indeed, it is already known that some viruses can accidentally produce defective virions in which there is no DNA packaged, and absence of certain scaffolding proteins are known to yield deformed capsid structures (Karhu et al. 2007, Earnshaw & King 1978, Black et al. 1994). Mutations in capsid proteins have been demonstrated to have an influence on the capsid size (see e.g. Mooney et al. 1987, Katsura 1983) and some retrotransposons assemble small, icosahedral virus-like particles (VLPs) that have been shown to exploit different T-numbers (T=3, T=4, T=7 and T=9, Al-Khayat et al. 1999). These notions together demonstrate that there is potential for variability in the virus progeny and thus

it is possible that viruses in their natural environments may randomly produce low amounts of virions with varying capsid architectures. P23-77, for example, might occasionally assemble virions of T=31 architecture due to the possible similarity between the natural T=28 and the "mutant" T=31 capsomer organizations.

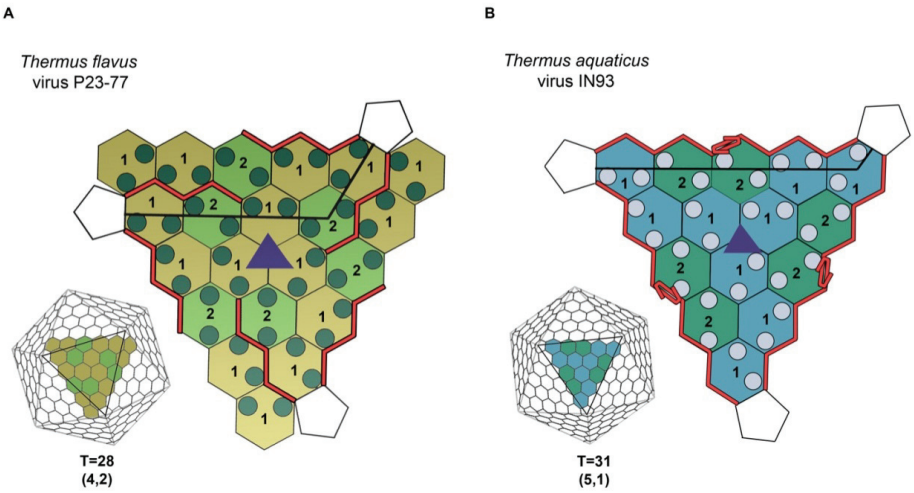

FIGURE 12 (A) A capsomer orientation in the T=28 virion of P23-77. (B) A model of the T=31 capsomer orientation in IN93. Red lines indicate the putative facet edges in the virion. The positions of decorating towers are depicted as circles over the hexamers.

Supposing that a virus DNA molecule lengthens significantly (through recombination, insertion or such), then the complete genome cannot fit into the original sized capsids and is thus forced to be packaged into one of the few capsids with higher T-number, if any. If only a partial genome is packaged, then some essential genes are likely to be lost. IN93 has been demonstrated to spontaneously acquire a novel insertion sequence, 1.2 kbp in length, from the host genome (Matsushita & Yanase 2009). Moreover, the IN93 related element in *Meiothermus silvanus* genome (MeioSilP1) has been invaded by transposable elements (II). Thus is appears possible that in nature the genomes of P23-77-like viruses may suddenly grow in size. It is also possible that the enlarged virus succesfully infects a new host. During such infection, any changes that promote the formation of larger capsids are highly favorable, as only the large capsids would most efficiently produce infectious virions. The increase in genome length and the subsequent infection of a larger virus could be seen as a bottleneck event, in which the mutated virus is, in a sense, the only survivor in the infected host. The scenario is illustrated in Fig. 13. To test the plausibility of this screnario, P23-77 genome could be artificially engineered to create an elongated DNA molecule and test whether it becomes packaged in some of the produced capsids. If this turns out to be possible, the evolution and adaptation

of this genome could be followed in order to understand the process of virus evolution towards larger genomes and capsids.

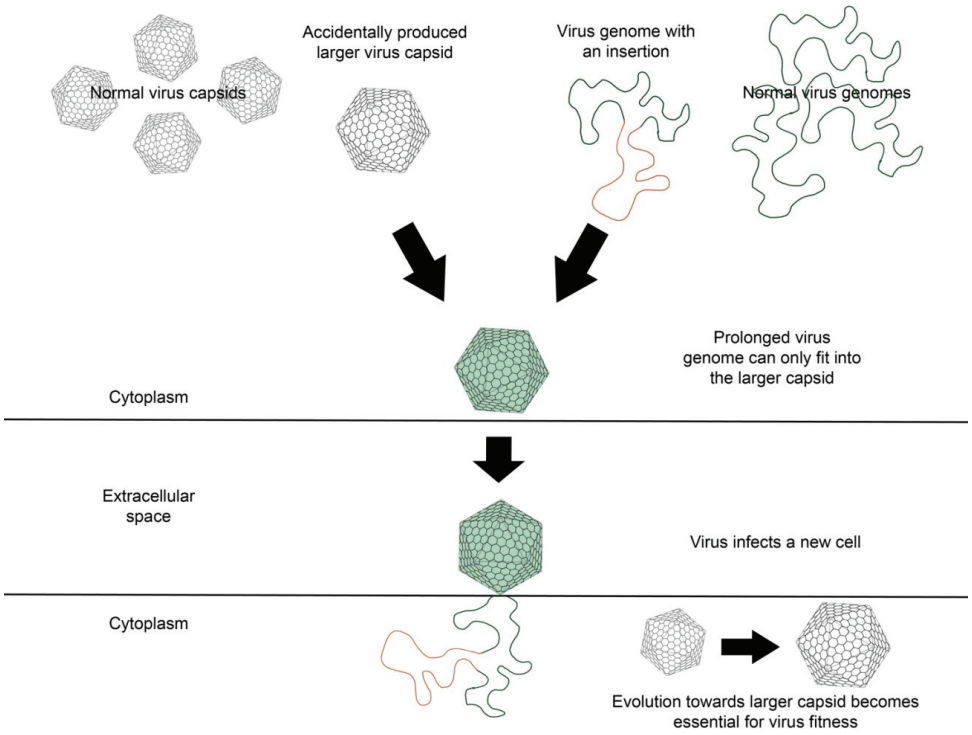

FIGURE 13 A presentation of the model suggesting one potential mechanism by which virus capsid might evolve in size.

In the previous section it was argued that the viruses belonging to the double-beta barrel lineage and the single beta-barrel lineage share a common ancestor, which most probably existed already in the primordial community of life before the establishment of the modern cellular domains. Formula (1) presents a way for roughly estimating the T-numbers of related viruses. The ancestor of these virus lineages probably had genes for a genome packaging ATPase, this being a roughly 650 bp long gene, and for a major capsid protein (preferably a single beta-barrel one), perhaps about 450 bp in length. The lengths are rough averages of the genes in the genomes of the contemporary viruses. Even the primordial version of the beta-barrel protein must have had enough amino acids for several beta-sheets and linker-regions which allow the sheets to form the barrel conformation. The primordial ATPase, on the other hand, had the domain for ATP hydrolysis along with the other necessary motifs and functions. Possibly the genes for these proteins have retained their size over evolutionary times. Anyway, the ancestral virus probably had a minimum genome of 1100 bp of double stranded DNA. Using either P23-77 (single beta-barrel) or PRD1 (double beta-barrel) structure as a reference, we get an estimated T-number

~4.5 for this ancestral virus. The two closest allowed T-numbers are 4 and 7. Probably the ancestor of this virus lineage used either of these T-numbers (or a greater number) for capsid architecture. However, the higher T-numer is more likely to be the correct one as the "rounding down" to T=4 would indicate that the genome must have been packaged into even higher density in the primordial virus than it is in its modern relatives. Nevertheless, the primordial virus might have built its icosahedral capsid out of 240 (if it used T=4) or 420 (T=7) major capsid protein subunits. Actually, if the genome was anything between ~750 bp and ~2800 bp in length, it would still be rounded to the closest T-numbers 4 and 7 (when either P23-77 or PRD1 was used as a reference structure). However, as the possible single beta-barrel protein and the ATPase take only 1100 bp out of the 2800 bp, then the virus genome might have had space for another 1700 bp of DNA. Therefore the genome encoding potential of a T=7 arranged ancestral virus could encompass additional genes that encode perhaps some genome replicating functions; a pentameric protein (perhaps responsible also for host recognition) located the vertices and/or the decorating protein of P23-77-like viruses. The modern versions of these structural genes fall easily within the range of 1700 bp, even if they were both present. This line of reasoning is, of course, very speculative, but it nevertheless suggests that the ancestor of the virus lineage might have been a relatively complex virus. This would, in turn, indicate that the last common ancestral virus of the beta-barrel lineages had already evolved within the system and thus did not emerge spontaneously.

5.3 Evolution of *Serratia marcescens* under selection of a T7-like phage

Infection by a lytic bacteriophage, like T7, inevitably causes the host cell death unless the host can somehow interfere or interrupt the virus life cycle. Nevertheless, the presence of a lytic bacteriophage in the environment rapidly causes the enumeration of virus-resistant bacterial phenotypes. The resistance usually comes with a price: often the cellular receptor needs to be modified or be lost altogether.

Given that viruses appear to be very ancient origin (Bamford 2003, Koonin et al. 2006, Villarreal & Witzany 2010), it is evident that such selection by virulent and also by temperate viruses among cellular populations has operated for billions of years. However, the effect of selection is arguably quite different between temperate and virulent viruses (Villarreal 2005), as, for example, the infection by a temperate virus can produce a surviving cellular lineage that has formed a symbiotic-like relationship with the virus (Ryan 2009). When a bacteriophage lysogenizes a bacterial cell, the cell (generally) becomes immune to further infections by the same virus type or by closely related viruses. Thus the lysogenic bacteriophages offer themselves the most rapid way for acquiring

the immunization. However, in lytic viruses it is often a host genome encoded characteristic that must change in order to the host to be resistant to the virus. Viruses always recognize some feature on the host cell surface for initiating the infection and this feature is likely to change under selection (VIII). We conducted one month long serial culture experiment to observe the changes in *S. marcescens* under selection of a strictly lytic PPV phage. The motility and biofilm formation were lowered in bacteriophage containing cultures. Moreover, the bacteria that evolved in absence of phages were more virulent in moth larvae. This suggests that the virus lowered the potential of the bacterium to reproduce in a different and complex environment. Some T7-like phages use type IV pili for host attachment (Chibeu et al. 2009). Type IV pili is responsible for flagella-independent moving mechanisms known as twitching motility. Therefore it is possible that PPV uses the same or related receptor. Thus the lowered motility under PPV-selection could be explained by emergence and enumeration of type IV pili-defective *S. marcescens* phenotypes. Indeed, type IV pili defective mutants are also incapable of forming biofilms (O'Toole & Kolter 1998) and have reduced infectivity (Comolli et al. 1999).

In section 5.6.2., it is argued that the absence of viruses should promote the evolution of characteristics that would not evolve in presence of viruses. This is indeed what was observed here with *S. marcescens.*

5.4 Plasmid-dependent viruses reduce horizontal gene transfer and provide a potential tool for fighting antibiotic resistances

Antibiotic resistant bacteria pose a significant risk to modern healthcare (Weigel et al. 2003). Plasmids are often responsible for encoding antibiotic resistances. Plasmids are also prevalent in many studied environments. Conjugative plasmids and antibiotic resistance genes appear to be common in soil (Malik et al. 2008). Saeed and colleagues studied 54 pathogenic Gram-negative bacteria, which were obtained from hospitals and labs, and demonstrated 46 % of them to carry transferable drug-resistance plasmids (Saeed et al. 2009). Smith et al. 2007, studied the drug resistances in commensal *E. coli* strains isolated from broiler chicken and came to the conclusion that the resistances are not explainable only by the prevalence of the resistant strains but mobile elements contribute significantly to bacterial resistances among these bacteria. Once a bacterium acquires a conjugative plasmid, it is very difficult to become free of the plasmid. Indeed, plasmids persist in bacteria in antibiotic-free medium for a long time in serial-culture experiments and their maintenance is almost costless to the host cell in terms of reproductive success (Dahlberg & Chao 2003). This suggests that the swiftly spreading, inherently incurable conjugative plasmids should be ubiquitously prevalent in bacterial isolates. However, many bacteria are also free of plasmids, proposing that in natural environments there might be an agent causing the plasmid loss. The selection by (PRD1-like) conjugative

plasmid-dependent bacteriophages could be one reason behind the loss of plasmids in natural environments.

Previously there have been few attempts to establish methods for curing plasmids from bacterial hosts. Bacterial exposure to certain small molecules, which mimic the factors that are used by plasmids to ensure their faithful distribution into the dividing daughter cells, appear to eventually cause the bacterial populations to lose some of the plasmids (Denap et al. 2006). In a follow up study Thomas and colleagues tested the ability of various aminoglycosides to cause the elimination of IncB-plasmid carriage within bacterial populations (Thomas et al. 2005). Generally about 20% to 50% of the plasmids were eliminated after 250 generations of constant exposure to these anti-plasmid molecules. However, I would argue that, while the plasmid-loss is promising, the rate is still too low and the process is too slow to be utilizable for practical applications. Plasmid-dependent bacteriophages appear to provide a more effective way for eliminating conjugative plasmids (V). While some studies suggest bacteriophages to serve as decent antimicrobial agents against certain bacterial species (Matsuzaki et al. 2003, O'Flaherty et al. 2005), PRD1-like phages could be used instead to treat some multiresistant infections (rather than treating selected bacterium).

PRD1 induced selection rapidly caused bacterial populations to decrease the antibiotic-resistant phenotypes. On average the resistance dropped in just 10 days to just few percent. If the serial culture experiments were initially supplied with plasmid-free bacteria, the percentage of drug-resistant conjugative plasmid-harboring bacteria dropped even more dramatically (data not shown). Most natural environments are likely to sustain a variety of plasmid-free bacteria and therefore PRD1-treatment (for even few days) might effectively reduce the amount of drug-resistant bacteria. Let's consider one practical example: Conjugative plasmids are responsible for some multidrug resistant strains of *Vibrio cholerae*, the causative agent of cholera (Pan et al. 2008). PRD1 is able to propagate on *Vibrio cholerae* given that the bacterium harbors a suitable plasmid (Olsen et al. 1974). Therefore it is possible that PRD1 and other plasmid dependent viruses could be used to combat, for example, drug-resistant cholera infections. PRD1-based applications could also be utilized to restrict the spread of drug-resistances in hospitals and other relevant environments.

However, regardless of the medical potential of plasmid-dependent bacteriophages, this study also provides potential insights to extend our understanding of microbial evolution. It has been argued that microbes used in experimental evolutionary settings can provide important information about the structures of adaptive landscapes (Colegrave & Buckling 2005). In this case, PRD1-selection had a variety of previously unknown effects on bacterial evolution. The sex-apparatus, which is encoded by conjugative plasmids, was disturbed and rendered defective by bacteriophage selection. Usually bacteriophages are treated only as agents that facilitate horizontal gene transfer between bacterial species (Miller 2001). In IV it was demonstrated that plasmid-

dependent bacteriophages provide an example of viruses that actually restricted the horizontal movement of genes. Given that bacteria are generally asexual organisms, it is possible that the four billion years of bacteriophage selection might have played a role in the prevalence of asexuality. Moreover, viruses are also known for their genome editing competence since they can introduce multiple novel genes into the cellular chromosome in a single evolutionary event (Witzany 2006). Plasmid-dependent bacteriophages provide an example of viruses with a different form of editing competence. Their presence in the environment can rapidly lead to the loss of large extra-chromosomal elements. Furthermore, these plasmids are apparently in a constant arms race with the viruses. Therefore the "host-parasite" coevolution occurs between a plasmid and a virus and, interestingly, not the traditional way between a host chromosome and a virus. Thus it can be a single batceriophage that is in a coevolutionary arms race with a multispecies community of plasmid-harboring bacteria.

5.5 Are the Diverse Crenarchaeal Viruses Survivors of the Early Virosphere?

Crenarchaea is a major kindom of the domain *Archaea*. All currently studied crenarchaeal species dwell in geothermally heated environments in temperatures that approach the boiling point of water. Viruses of crenarchaea have unusually diverse variety of capsid morphotypes (Rachel et al. 2004, Prangishvili et al. 2005) and their genomes are very different, indicating that they might be independently emerged viruses (Prangishvili et al. 2006).

If the "virus first" model for explaining the origin of viruses (see e.g. Koonin et al. 2006) is the correct one (as it seems to be, at least for some of the viruses) and the vast majority of viruses have retained their morphology throughout the four billion years of evolution (like PRD1 and Adenoviruses, head-tail viruses, reovirus-like viruses and some pleomorphic viruses, Pietilä et al. 2009), then diversity of virus morphotypes would have been highest at some point during the early evolutionary period of life. If new morphotypes emerged only rarely, then the morphological diversity might be mostly decreasing. This is because the *de novo* emerged viral lineages go extinct. (However, it must be noted that some viruses have developed novel capsid morphotypes from a well-established ancestor, like ascoviruses. But in the case of ascoviruses the common ancestry of ascoviruses and icosahedral nucleocytoplasmic large DNA viruses is still recognizable by genomic analyses, Stasiak et al. 2003).

What could cause widespread extinctions among prokaryotes so that it also affected the diversity of their obligate cellular parasites (i.e. viruses)? Extinctions are common in Earth's history (Raup & Sepkoski 1984, Raup 1986, Rohde & Muller 2005). The early life forms were under constant celestial bombardment by meteorites and comets (Gogarten-Boekels et al. 1995). The so-

called Late Heavy Bombardment period (LHB) was an era in Earth's history (about 4.1 to 3.8 billion years ago) during which large meteorites frequently hit Earth (Nisbet & Sleep 2001, Zahnle & Sleep 2007). These meteorite-impacts released vast amounts of energy and possibly even boiled the oceans (Alvarez 2003). LHB ended about 3.8 billion years ago, but great meteorites have hit Earth numerous times also thereafter (Alvarez & Asaro 1990). Several studies date the origin of life to have occurred about 4 billion years ago (see e.g. Battistuzzi et al. 2004), thus microbes have inevitably faced more or less widespread events of decimation due to celestial impacts. Hyperthermophiles, however, would not be sensitive to rising global temperatures due to the characteristics of their natural habitats. Boiling oceans would not represent a catastrophe for a population of microbes that already thrive in a boiling geothermal environment (Kawashima et al. 2000). Moreover, studies also suggest that Earth was entirely surrounded by an icesheet during the so-called 'snow ball' Earth events (Hoffman et al. 1998, Donnadieu et al. 2004). Geothermally heated regions, however, would have remained warm throughout the glaciation periods, as sunshine never played a significant role in their warming. Given this line of reasoning, it would be logical to assume that the viruses of microbes of geothermally warmed areas would not run out of hosts during extinction events. This, in turn, would give them a better chance in surviving the catastrophes (see Fig. 2 in VIII). Therefore, it is possible that the diverse hyperthermophilic crenarchaeal viruses resemble more completely the original morphological diversity of the early virosphere. If the early biosphere actually consisted of a variety of unique viruses, then the conditions of precellular evolution of life must have been something that supported the emergence of several viral lineages. Including the diverse virosphere into our models concerning the early evolution of life might not tell us exactly what kind of a system the primordial living community was, but potentially it can help us narrow the range of potential hypotheses.

5.6 Viruses and the Early Evolution of Life

5.6.1 Viruses and the evolution of proto-cells

In 1994 Koch argued that there is no trivial spontaneous way for polynucleotides to be introduced into living cells and thus transfer of genes from one "organism" to another did not occur among the primordial life forms. I would argue that viruses are exactly something that transfers polynucleotides from one cell to another. Thus, even if genes cannot be spontaneously introduced to cells, there might have been primordial, genetically encoded agents making the genetic transfer possible. Vetsigian and colleagues argued in 2008 that the universal genetic code is the result of the innovation sharing within the primordial community of life. Perhaps the emerging virus-like entities were one of the agents that spread the innovations between protocells.

In 2005 Lehto and Karetnikov argued that the present-day RNA-viruses and viroids might resemble the early replicators in their molecular and functional properties. Indeed, in VI it was shown that within a simulated compartment-matrix (inhabited by ribozyme-like agents) virus-like genes (or agents) are favored due to their ability to transfer genetic innovations between proto-cells.

However, as the system keeps evolving, the genetic innovations would slowly accumulate into proto-cells and thus the cells became less relied on extracellular resources (like e.g. the abiotically formed amino acids) on replicating and producing cellular components. It is possible that some of the virus-like entities turned into pure resource consuming parasites (alike modern viruses), because these parasitic phenotypes were able to outcompete the "beneficial" innovation-sharing viruses in terms of reproductive success. This might have caused the proto-cells to favor any trait that isolated them from the community since isolation probably protected the cells from some virus infections. Plant cells, for example, are much harder to be invaded than animal cells, because of the thick and impenetrable cellulose wall. Therefore most plant viruses need help from, for example, insects to pass the cellular barrier (Villarreal 2005). One possible course of evolution of isolation would have been the emergence of cell walls. The walls could have prevented any virus-parasites from entering the proto-cell. We demonstrated in IV that viruses (exploiting the genetic exchange apparatuses of cells) effectively removed the ability of bacterial cells to horizontally exchange genetic material with one another. Therefore it seems plausible to expect similar forces to have worked on primordial Earth. If easily intruded proto-cells became invaded and demised by viral parasites, we should expect the hard-to-invade proto-cells to prevail. Eventually the population of proto-cells would encompass mostly of isolated cells. However, viruses would be bound to evolve to invade the more isolated cells and thus the cycle to favor even more isolated or otherwise virus-resistant cells would be repeated. At some point, the cells would acquire a certain level of independency from their present environment. Perhaps the first independent and free-living bacteria and archaea emerged by this tradition through a repeated rounds of virus-induced selection. It is difficult to study the plausibility of such a scenario, but in the future sophisticated computer simulations might be able to test the hypothesis.

5.6.2 Decrease of virus diversity after extinction events and its possible effect on host evolution

In 1995 Newman and Roberts suggested that species tend to evolve towards being more sensitive to climate changes and other environmental stresses because they adapt to cope only with their current environmental conditions. Therefore rapid changes in climate are more likely to cause widespread extinctions if there has been sufficient time for species to evolve after the previous climate change. Meteorite impacts can rapidly change the global climate and thus cause mass extinctions (Pope et al. 1997). Permian extinction,

for one, appears to have drastically decreased the number of species 252 million years ago (White 2002, Mundil et al. 2004). Throughout the evolutionary history of life on Earth, viruses have been affecting the evolution of microbes (Comeau & Krisch 2005). Viruses can maintain host diversity by favoring variations of the dominating species (Weinbauer 2004, Weinbauer & Rassoulzadegan 2004). Thus virus imposed selection causes natural selection to often favor attributes that simply help them to avoid infections. Viruses are also constantly under the pressure to keep up with their evolving hosts and therefore a homogenous population of viruses develops a variety of phenotypes that can infect a wider range of hosts (Weitz et al. 2005). The longer the virus population exists, the more cellular receptors they exploit for recognizing potential hosts. Within a matured community, it is possible that majority of mutations are favored just because they make the species less susceptible to virus infections. However, if viruses were absent (or there were much fewer viruses), then natural selection could instead favor the evolution of other characteristics. This is what we observed in our evolution experiments with *S. marcescens* (see section 5.3).

Now, let's consider a hypothetical ecosystem in which a rather stable population of 100 cells thrives. This population can maintain only a certain variety of obligatory intracellular parasites (viruses). Let's say, for example, fifty different viruses that all use different surface component as a cellular receptor. If an extinction event occurs and the population of 100 cells was reduced to two cells, the remaining surviviros might be able to support only a single virus. The other forty-nine viruses would eventually go extinct because of the lack of hosts. The surviving virosphere would be able to recognize only a single surface component. The cells would recover from the event and eventually replace the ecological niches of the rest ninety-eight cells. Importantly, the viruses would be less diverse and could infect fewer host phenotypes than they did before the extinction event. Therefore there might be a period of time after global catastrophes that natural selection mainly favors other than virus-resistance traits among cellular organisms. This, in turn, would perhaps accelerate the rate at which cellular organisms come up with novel evolutionary innovations. The scenario is presented in Fig. 14.

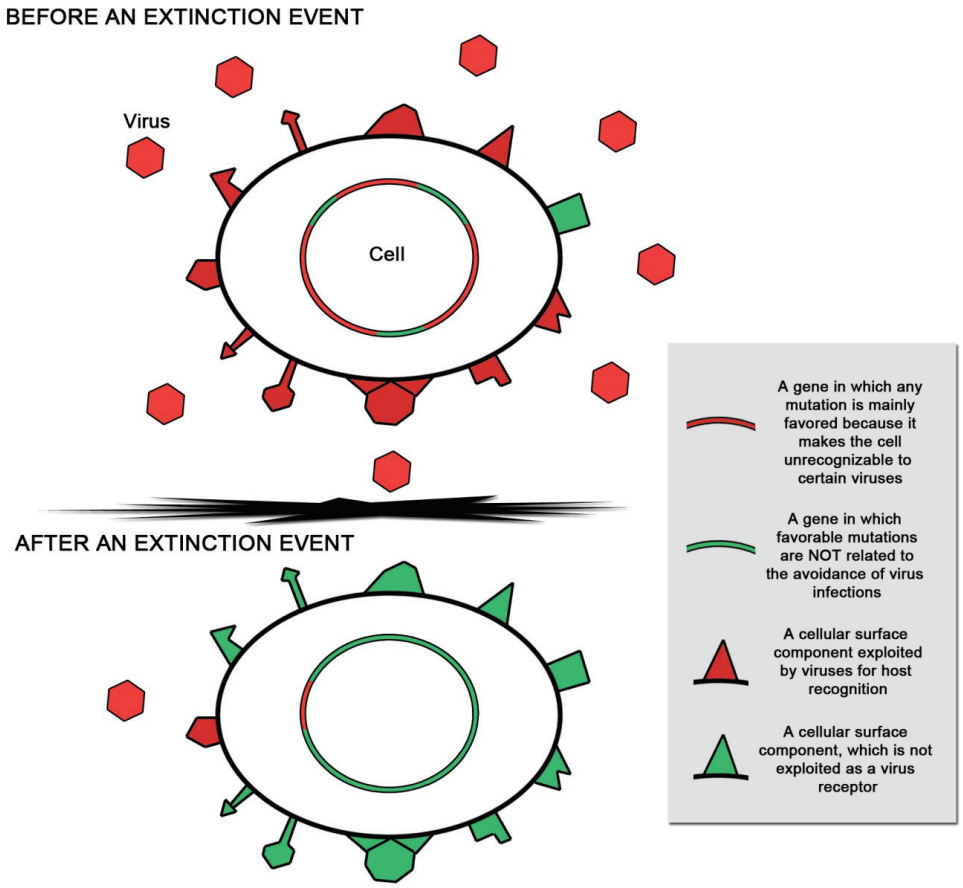

FIGURE 14 A model depicting the varieties of viruses before and after an extinction event. Before the event, the viruses should have evolved to recognize most of the useful receptors on the potential host cells. After the extinction event, the number of viruses would decrease along with the number of hosts. The fewer viruses should mean that there is also a decrease in the number of cellular surface components that are exploited as virus receptors. This, in turn, might relieve the selection within the ecosystem to favor traits that are not always directly related to the avoidance of viral infections.

5.6.3 Endomembrane compartments in emerging eukaryotic cells

Forterre and Prangishvili wrote in 2009,

> "Jalasvuori and Bamford, for instance, suggested that protoviruses already originated at the onset of the first age of the RNA world, when a replicative RNA bound to the membrane of a primitive but large vesicle had acquired the capacity to trigger budding of small vesicles that were then able to fuse with another vesicle (infection). Thus, a better knowledge of the mechanism of production of modern vesicles could possibly shed light on the origin of viruses."

Forterre and Prangishvili are very kind in acknowledging our ideas. I suggest that one possible approach for improving the knowledge on the origins of vesicle-manipulating genes would be to determine the possible evolutionary forces that drove the emergence of vesicle-forming mechanisms in Eukaryotes. The operation of the endomembrane system of Eukaryotes essentially involves constant vesicle fusions and formations, as there is a continuous back and forth traffic of vesicles between the cell surface and various organs. In this respect the eukaryal cell resemble a community of membrane-bound proto-cells with vesicles travelling constantly from one compartment to another.

What do we know about eukaryal endomembrane system? Membrane bound organs such as Golgi apparatus, endoplasmic reticulum, nucleus and mitochondria essentially compartmentalize eukaryotic cells. Early eukaryotes already had a complex endomembrane system (Dacks & Field 2007) and all modern eukaryotes have evolved from a mitochondria-bearing ancestor (Embley et al. 2003, Embley & Martin 2006). Some (but not all) eukaryal genes show relatedness to archaea and bacteria (Hartman & Fedorov 2002, Horiike et al. 2001, Rivera & Lake 2004, Podar et al. 2008, Koonin 2006, Yutin et al. 2008). Viruses were also proposed to have played a role in the origin of nucleus and other eukaryal features (Bell 2001, Koonin et al. 2006, Villarreal et al. 2000, Forterre 2006, Takemura 2001, Villareal 2005). Certain viruses have linear, eukaryote like genomes and they replicate their DNA in a similar manner. Some viruses possess eukaryote specific DNA-repairing enzymes. Many viruses have features with functional similarities to nuclear pores and some viruses extrude messenger RNAs from the virion in a somewhat similar way with nucleus. There are some putative simple endomembrane system homologues in prokaryotes (Dacks & Field 2007). Bacteria *Planctomucetes* have membrane-enclosed nucleoid, somewhat similar to a eukaryotic nucleus (Fuerst 2005), and in archaeon *Ignicoccus islandicus* small vesicles can be observed in electron microscope micrographs (Rachel et al. 2002). Some parts of ER translocational system of eukaryotes are also present in prokaryotic SRP/SecY translocation system (Gribaldo & Cammarano 1998) and bacterial dynamin-like protein among some other features have been described (Low & Lowe 2006). Yet, generally features of any resemblance to inner lipid compartments of eukaryotes are absent in prokaryotes.

The last common eukaryote ancestor already possessed endomembrane system of high complexity and this system was suggested to have formed autogenously from pre-existing building blocks of domains and motifs (Dacks & Field 2007). Indeed, endoplasmic reticulum, nuclear envelope, sophisticated transporting machinery, Golgi-apparatus and dedicated vesicles are novel evolutionary steps in cellular development. Did some of the domains and motifs emerge in the genomes of virus-like parasites? Viruses must have been manipulating the entry and exit through cellular membrane much before the emergence of modern eukaryotes and thus they probably encoded some suitable genes for facilitating the vesicle-traffic. Perhaps the origin of endomembrane system was not a peculiar phenomenon, which occurred

overnight, but instead a logical consequence of the evolutionary dynamics within a community of various living entities.

Most of the current debate addressing the origin of eukaryotes revolves around the question whether it was an archaeaon and a bacterium that fused to produce the eukaryote or if it was some form of proto-eukaryote and bacterium that formed the eukaryal cell. Genetic evidence alone might not provide a sufficient model for explaining why the origin of eukaryotes occurred in the first place. Instead we should also consider the contribution of the potential habitat of the proto-eukaryotes in the evolution towards the modern eukaryotic cell. In any case, being relevant or not, the origin of eukaryotes had to occur *somewhere* and all life forms on Earth are adapted to live in their particular *somewhere (*with the other inhabitants).

5.7 Viruses and the Life in the Universe

Finally I would like to make some more general arguments about studying the potential existence of life elsewhere in the Universe. The key question, of course, is, whether or not there exists extraterrestrial life. Or is the consideration of such matters waste of time altogether? I have personally noticed that any attempts of providing even the tiniest contributions to this theme appear to be some sort of heresy or pseudoscience to certain biologists. One of their main arguments is always the notion that "*We have no examples of extraterrestrial life, so what is it exactly that you are studying?*" Is it science to try understanding something that might not exist at all? Most biological studies are examining a single organism or a single process in order to provide models and examples that apply for majority of life on Earth. Should we also try to answer the follow-up question: How well do these models apply to other (although hypothetical) living systems in the Universe?

What is it that the so-called astrobiologists (i.e. those who are interested in asking the question whether or not there is life out *there*) are studying? Biology emerged as a subject that attempted to make sense to the living world around us. Astrobiology is not something that emerged to explain new observations like microbial cells under microscope (microbiology) or even tinier disease causing particles (virology) (Dartnell 2007). Instead, we know that there is life in this Universe (on a pale blue dot in space, known as Earth) and there are obviously countless other Earth-sized planets in the Cosmos (Lissauer 2002, Selsis et al. 2007, Mayor et al. 2009). Is it possible to find life from some of these planets (or perhaps elsewhere)? What is it that life requires? Are there same main themes governing the emergence and/or evolutionary pathways of life everywhere in the Universe? Can we draw any uniform conclusions of life by observing this one example of life here on Earth? Evolution itself is something that is also true for various essentially non-biological things like culture and economy. Any reproducing alien life forms out there are inevitably slaved by the laws of evolution. But are there any other aspects in our terrestrial life that

could also be true for the extraterrestrial ones? For example, all cellular life uses DNA for storing genetic information. Should we expect to find DNA from the cells of other independently emerged life forms? Perhaps not, but as the ability of DNA and RNA to direct the base pairing of the complementary strand is very essential for all life on Earth, it could be possible that extraterrestrial life similarly uses this principle for guiding the synthesis of the genetic progeny. There is rather plausible evidence suggesting that life on Earth could have originated within a geothermally heated hydrothermal vent (Koonin & Martin 2005). Is the existence of hydrothermal vents a prerequisite in order to expect life to be able to emerge? Or more likely, should we ask whether it is water, the tiny compartment matrix and the concentration of essential "organic" molecules that life requires? Many different geological and chemical processes could possibly produce these suitable conditions.

The history of biology is saturated with examples of attempts of finding something that apparently could exists but what just have not yet been discovered. The fossil record lacked many key intermediates between the land-dwelling and waterborne primates when the theory of evolution was first established (Darwin 1857). Since then, many of these gaps have been filled and the theory of evolution can indeed predict their existence. Another example is the discovery of DNA polymerase. It eventually became apparent to Arthur Kornberg that there might be an enzyme catalyzing the polymerization of a DNA strand from triphosphate nucleotide monomers (Lehman 2003). It required determined work to find this enzyme (Kronberg et al. 1956). Without the dedication to seek for something that was not yet discovered but which possibly existed, the more profound understanding of living organisms would have been postponed (along with Kornberg's Nobel prize). Almost everyone on Earth is somehow thrilled about the question whether we are alone in this Universe or whether there is something living *out there*. As our knowledge of life increases, we can better understand where life might exist and what it might be alike. I believe that this is what astrobiology is all about.

Furthermore, I would argue that considering the possible existence of alien viruses might help us extend our comprehension of life in general. Are there viruses on other planets? Are the hypothetical extraterrestrial living beings infected with viral parasites similarly to the life on Earth? At first these questions might seem pointless since viruses are dependent on living organisms for reproduction. If we know nothing of the extraterrestrial life, why should we ask questions about their parasites? One could argue that it would be of equal importance to ponder whether extraterrestrial life also drives cars on the right hand side of the road or listens to heavy music. However, I suggest that considering the possibility of the existence of extraterrestrial viruses rises above of being just a curiosity. Let's take an example within our own solar system. Many planet-landing probes have been and will be designed to recognize biosignatures (i.e. signs of life). There are at least ten times more viruses on Earth than there are cellular organisms. If viruses were expected to infect organisms on other planetary bodies (like Mars), then any attempts to

detect this life on-site (similarly to e.g. ESA's Aurora ExoMars Life Marker Chip; Parnell et al. 2007) would be likely to study samples in which viruses are present. It would be important to, at least, acknowledge the possibility. The designers of the detectors should ask whether their apparatuses are capable of detecting viruses. Currently this does not appear to be the case. The antibody approach used in Life Marker Chip had no antibodies for recognizing any virus components but only many cellular ones, thus Martian life might go unnoticed as no one considered the possibility that only viruses got into the studied soil sample. Viruses were not even mentioned in the paper published on the life detection goals of ExoMars. The presence of viruses would indirectly indicate the presence of cells in the given environment. We tested whether the antibodies against the conserved coat protein of bacteriophage PRD1 (Fig. 1 in IX) were able to bind the coat proteins of the distantly related viruses of Bam35 and AP50 (IX). The two virus proteins probably diverged from one another already about 3.7 billion years ago (unpublished data). Therefore the potential viral relatives on Mars could be equally divergent. However, the anti-P3 antibodies did not recognize the coat proteins of AP50 nor Bam35. Our single attempt is not enough to conclude that antibodies cannot be used for detecting viruses that share a common ancestor with the viruses on Earth. Antibodies are, in any case, used widely in different diagnostics to identify the presence of viruses in clinical and other types of samples (Michaud et al. 2003). However, recognizing distant relatives might still prove impossible. Nevertheless, experimental studies also suggest that antibodies represent clear astrobiological potential (Schweitzer et al. 2005). Furthermore, any attempts to detect nucleic acids or proteins might also be able to recognize viruses and thus they seem as good choices for detecting any Earth-related life within our solar system. However, amino acids are known to be present also in many non-biological systems. It has also been argued that any extraterrestrial sequence data is very likely to be due to detector contamination (Poole & Willerslev 2007). Thus extreme caution needs to be exercised in designing and studying any potential extraterrestrial life.

Aside of the direct detection of extraterrestrial life, viruses can also help us understand how alien life forms develop. Viruses emerged early in the life's history and since then they have affected the evolution of life in multitude of ways (Villarreal 2005, Villarreal & Witzany 2010). Could it be that the origin of viruses was not just a coincidence but instead it was an essential part of the early evolution of life (like suggested in VII, computationally studied in VI and discussed in the previous sections)? Are we thus bound to find viruses from any naturally emerged ecosystems in the universe? Moreover, some scientists have speculated that life could travel from one planet to another within meteorites. We know that some Martian rocks have ended up here due to the impact events that have launched some of the Martian surface particles to space (Davies 1996). Probably there are also rocks from Earth resting on the surface of Mars. Highly resistant endospores of certain bacterial species have been tested to be optimal forms of life to survive these interplanetary voyages (Horneck

1981, Horneck 1993, Horneck et al. 1994, Dose & Klein 1996, Horneck et al. 2001, Bruchell et al. 2003). What if the planet-contaminating bacterial endospore did not contain any proviruses inside? If viruses only (or mostly) emerged in the hypothetical primordial compartment matrix of simple replicators and later proto-cells, then the life on the contamined planet might never encounter viruses. Given the 10^{32} viruses on Earth and the suggested 10^{25} infections occurring per minute (Wommack & Colwell 2000, Wommack et al. 1992, Pedulla et al. 2003), we can make only wild guesses how such a virus-free ecosystem might evolve in the long run. Perhaps viruses do not have that significant of an effect after all. Or perhaps everything would be very different in the alien biosphere.

6 CONCLUSIONS

The main conclusions of the thesis are:

- The group of icosahedral, inner membrane containing viruses infecting thermophilic bacteria and halophilic archaea form a viral lineage. All the members of the lineage share three common genes encoding: an ATPase, a small major capsid protein and a large major capsid protein. The common ancestor of this lineage existed perhaps before the bacterial and archaeal cells separated into the two domains of life.
- The ATPases, icosahedral shape and the inner membrane of the P23-77-like viruses are common to the previously demonstrated lineage of viruses with double beta-barrel major capsid protein. Possibly the double beta-barrel viruses and the P23-77-like viruses separated into their distinct branches already in the primordial pre-LUCA community of life.
- Presence of plasmid-dependent viruses causes bacterial populations to favor plasmid-free cells. This demonstrates a novel form of genome editing competence in viruses as only few days of bacteriophage selection led to the loss of large extra-chromosomal elements from the cells. Moreover, the virus-selection suggests that conjugative plasmid-dependent viruses might have played an important role in the evolution of asexuality in microbes. Plasmid-dependent viruses could also be used to combat pathogenic drug-resistant bacteria.
- Viruses might have participated to the evolution of the primordial community by facilitating the innovation sharing between proto-cells. Later viruses might have promoted the isolation of the proto-cells and thus made it possible for the proto-cells to evolve towards independency.

- The diverse morphotypes of viruses infecting hyperthermophilic *Crenarchaeota* might be a result of better survival ratio of the virus hosts during meteorite impacts and other global catastrophes. If most of the virus morphotypes emerged already during the early biosphere, then the current diversity of crenarchaeal viruses may reflect, for some parts, the emerging virosphere.

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The four billion years of evolution have made us quite selfish as far as the survival of our own genes is concerned. Usually these are just words that we biologists repeat like a spiritual mantra. However, it is so enlightening (as a biologist) to actually feel all the billions of years in the love that you have for your own children. Eemil and Lilja (and any progeny that I may have in the future) you are so very dear to me.

YHTEENVETO (RÉSUMÉ IN FINNISH)

Virukset ovat muinaisia loisia, jotka ovat vaikuttaneet nykyisten ja varhaisten elämänmuotojen kehitykseen.

Tässä väitöskirjassa esitetään todisteita ja malleja, joilla pyritään parantamaan vallitsevia käsityksiä siitä miten virukset ovat syntyneet ja miten virusten syntyprosessi on saattanut vaikuttaa solujen alkumuotojen kehitykseen.

Virukset ovat pieniä proteiinikuoren ympäröimiä loisia, jotka pystyvät lisääntymään ainoastaan solujen sisällä käyttäen hyväksi solujen koneistoa ja resursseja. Vasta viime vuosina on saatu ensimmäiset todisteet siitä, että virukset ovat hyvin vanhoja – ne ovat syntyneet todennäköisesti jopa paljon ennen kaiken solullisen elämän kantaisää noin neljä miljardia vuotta sitten. Miten voi olla mahdollista, että täysin soluista riippuvaiset virukset voivat kehittyä ennen kuin nykyisenkaltaisia soluja edes oli olemassa?

Väitöskirjatutkimuksessa löydettiin täysin uusi virusperhe, joka näyttää syntyneen hyvin varhaisessa vaiheessa elämän alkutaipaleella. Nämä kuumissa lähteissä elävien bakteerien virukset ja hyvin suolaisissa järvissä (ja muissa ympäristöissä) elävien arkkien virukset ovat ainoat toistensa selvät sukulaiset. Niillä on uniikki kuorirakenne, joka muodostuu tynnyrimäisistä pääkuoriproteiineista ja niitä tukevista koristeproteiineista. Tämän niin kutsutun yksöisbetatynnyrivirusperheen isäntäsolut ovat toisilleen kaukaisinta mahdollista sukua maapallolla, joten lienee todennäköisintä että jossakin alkumaailman olosuhteissa ennen isäntäsolujen erkanemista syntyi tämän virussuvun kantaisä. Yksöisbetatynnyrivirukset ovat sittemmin tiiviisti seuranneet isäntäsolujaan neljän miljardin vuoden ajan. Lisäksi tutkimuksessa huomattiin, että tämän virusryhmän pääkuoriproteiini ja perimänpakkausjärjestelmä vaikuttavat olevan etäistä sukua ensimäiselle koskaan löydetylle ennen solujen esi-isää syntyneelle viruslinjalle, ns. kaksoisbetatynnyriviruslinjalle. Hyvin alkukantainen virusryhmä lienee siis jakautunut kahdeksi eri sukulinjaksi jo ennen ensimmäisiä todellisia soluja. Tämä viittaa siihen että alkumaailman esisolullisen yhteisön on täytynyt olla tarpeeksi suuri, jotta virukset ovat voineet eriytyä useiksi linjoiksi.

Mutta miksi tärkeimmät virusgeenit näyttävät syntyneen alkumaailman olosuhteissa, mutta eivät enää sen jälkeen? Väitöskirjatutkimuksessa suunniteltiin ja toteutettiin laskennallinen simulaatiosysteemi, jolla voidaan tutkia virusgeenien hyödyllisyyttä alkumaailman alkukantaisissa geeniyhteisöissä. Simulaatiotulosten perusteella viruskuoriproteiinien esimuotoja muistuttavat tekijät auttoivat alkuyhteisöjen keksintöjen levittämisessä esisolujen välillä. Tämä nopeutti evoluutiota systeemin sisällä, koska uudet geneettiset keksinnöt olivat kollektiivisesti koko yhteisön käytössä. Erilaisia systeemeitä vertailtaessa huomattiin että virusgeeneistä vapaat järjestelmät hävisivät tehokkuudessa virusgeenejä sisältäville yhteisöille. Ensimmäiset esi-virukset ovat siis saattaneet olla hyvin tarpeellisia alkuelämän kehitykselle.

Virukset ovat myös saattaneet ajaa ensimmäisten solujen itsenäistymistä. Alkusolujen on täytynyt puolustautua virusinfektioita vastaan ja täten esimer-

kiksi soluseinien kehittyminen ja muut seikat, jotka ovat estäneet virusten tunkeutumisen soluun, ovat edesauttaneet esisolujen eristäytymistä muusta alkuyhteisöstä. Väitöskirjatutkimuksessa testattiin seksuaalisten bakteereiden kehitystä virusten läsnä ollessa ja huomattiin että virukset tehokkaasti vähensivät bakteerisolujen välistä geeninsiirtoa. Täten myös alkusolut ovat mahdollisesti eristäytyneet muista soluista samankaltaisen virusvalintapaineen alla.

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