Immunodetection of VP11, the minor capsid protein of thermophilic bacteriophage P23-77

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Preface

The experiments of this thesis were conducted at the University of Jyväskylä, Department of Biosciences in the division of cell and molecular biology under Jaana Bamford's group "Genetics, Assembly and Evolution of Viruses". I wish to thank my supervisor Alice Pawlowski, who guided me with the laboratory experiments and patiently helped in the writing process. In addition, I want to thank Petri Papponen and Sari Mattila, who advised me with the electron microscopy experiments and other laboratory practices. Thanks to Jaana Bamford, who gave me the opportunity to work in her group. For the thesis, a fund from Betty Väänänen foundation was received, of which I'm really grateful. Finally, I want to thank my family and friends who supported me during the writing process.

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Anni Moilanen

University of Jyväskylä **Abstract of Master´s Thesis**

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Abstract:

VP11 is a minor capsid protein of the thermophilic bacteriophage P23-77 and comprises the icosahedral capsid mainly with two major capsid proteins (MCPs) virion protein (VP) 16 and VP17. The exact localization and function of VP11 is unknown. Yet, previous studies suggests that VP11 is located either between the protein capsid and the internal lipid membrane or occupies the capsid vertices on the outer side of the capsid. The aims of the study were to optimize a western blot method for the detection of VP11, estimate the copy number of VP11 in the virus particle and study the localization of VP11 by using immunolabelling and transmission electron microscopy (TEM). Western blots were performed with purified virus samples in a semi-dry transfer system with varying immunolabelling conditions. The copy number of VP11 was analyzed based on band intensities in denaturing polyacrylamide gels. P23-77 was cultured by using *Thermus thermophilus* as a host and purified by rate zonal and equilibrium centrifugation. Purified virus particles were immunolabelled for TEM. The optimization of the western blot method succeeded, specific signals were received after suitable immunolabelling conditions were found. In addition, the western blot results imply that VP11 exists as a dimer in the P23-77 virion. The copy number of VP11 was estimated to be approximately 147 copies. Both findings indicate that VP11 is not the penton protein occupying the capsid vertices. Supporting this theory, preliminary studies with TEM suggest that VP11 does not exist on the outer side of the capsid. Rather, VP11 is located underneath the protein shell as a dimer. Due to its proposed location and other qualities, VP11 might have potential in future biochemical applications.

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VP11 on termofiilisen bakteriofagin P23-77 kapsidiproteiini ja se muodostaa ikosahedraalisen kapsidirakenteen yhdessä pääkapsidiproteiinien, virusproteiini (VP)16 ja VP17, kanssa. VP11 tarkkaa paikkaa ja toimintaa kapsidissa ei tiedetä. Aiemmissa tutkimuksissa VP11 on arveltu sijaitsevan joko kapsidikuoren alapuolella tai kapsidin ulkopinnalla piikkirakenteissa. Tutkimuksen tavoitteena oli optimoida VP11 havaitsemisessa käytettävä western blot –menetelmä, arvioida VP11 määrää kapsidissa ja tutkia VP11 sijaintia vasta-ainevärjäyksen ja transmissio elektronimikroskopian (TEM) avulla. Western blot –menetelmän optimoinnissa käytettiin puoli kuivaa (engl. semi-dry) menetelmää vaihtelemalla vasta-ainevärjäysolosuhteita. VP11 määrää kapsidissa analysoitiin denaturoivan polyakryyliamidigeelin juovien intensiteetteihin avulla. P23-77 kasvatettiin isäntäbakteeri *Thermus thermophiluksessa*, jota seurasi kaksivaiheinen viruspuhdistus. Puhdistettu virus vasta-aineleimattiin ja kuvattiin TEMilla. Western blot –menetelmän optimointi onnistui, menetelmä antoi tarkan signaalin optimaalisten vasta-ainevärjäysolosuhteiden löydyttyä. Lisäksi western blot –tulokset viittasivat siihen että VP11 on rakenteeltaan dimeeri. VP11 kopiomäärän arvioitiin olevan 147, joka viittaa siihen että VP11 ei ole osallisena viruksen kapsidin piikkirakenteissa. Alustavat TEM kokeet tukevat sitä että VP11 ei sijaitse kapsidin ulkopinnalla. Saatujen tulosten perusteella VP11 sijaitsee viruskapsidin sisäpuolella dimeeri-rakenteena. Proteiinin sijainnin ja muiden ominaisuuksien vuoksi VP11 saattaa olla hyödyllinen tulevaisuuden bioalan sovelluksissa.

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Abbreviations

VP= Virion protein

MCPs= Major Capsid Proteins

TM= *Thermus* media

TEM= Transmission electron microscopy

PFU= Plaque forming units

OD550= Optical density at wavelength 550 nm

CFU= Colony forming units

MOI= Multiplicity of infection

PEG= Polyethylene glycol

1x-purification = Rate zonal centrifugation

2x-purification = Equilibrium centrifugation

PTA= Phosphotungstate acid

FCS= Fetal calf serum

PBS= Phosphate buffered saline

SDS-PAGE= sodium dodecyl sulfate polyacrylamide gel electrophoresis

PVDF= Polyvinylidene fluoride

1. Introduction

1.1 Viruses in extreme environments

Extreme environments were first thought as places where no life can be found. The conditions met in these surroundings may be for instance, high temperature or extremely low or high pH (for review see Canganella and Wiegel, 2011). Bacteriophages are viruses infecting bacterial cells (Breitbart et al., 2004). Organisms living in extreme conditions are called extremophiles and they are classified considering their growth requirements. For instance, halophiles include microbes inhabiting extreme salt concentrations (over 1 M) and thermophiles include microbes, whose optimal growth temperature resides between 60 and 80 °C. Hyperthermophiles thrive in environments over 80 °C (Horikoshi et al., 2010). In contrast to the initial belief, extreme environments have been shown to possess abundant microbial life (Breitbart et al., 2004; for review see Canganella and Wiegel, 2011; Wall et al., 2015). For example, hot springs contain a high number of viruses and they have been estimated to have an important role in the nutrition and carbon cycles of these environments. In addition, thermophilic viruses are the main predators of procaryotes in high temperature conditions (Breitbart et al., 2004). In general, viruses residing in extreme environments are not yet studied thoroughly, partly due to the fact that extreme conditions are hard to maintain in laboratory conditions (Kim et al., 2008).

Viruses occupying extreme environments have adaptations for surviving in the harsh conditions (Cox et al., 2010; Pietilä et al., 2013). For instance, due to the strong fluctuations of the temperature in the extremely hot environment, the proteins of thermophilic viruses are considerably thermostable (Bull et al., 2000; Jaatinen et al., 2008). In addition, haloarchaeal viruses may remain inactive during low-salinity periods when the host cells are not viable. When the salinity of the environment increases, the viruses become more infective as their host's viability grows (Pietilä et al., 2013). Due to the adaptations, extremophilic viruses might possess unique qualities, which may have potential in biochemical and industrial applications (Song and Zhang, 2008; for review see Canganella and Wiegel, 2011; Keen, 2015). Especially thermophilic microbes have been studied in the search of new thermostable enzymes. For instance, the RNA polymerase used in the transcription step of PCR reaction was discovered from *Thermus aquaticus (*Air

and Harris, 1974; Frock and Kelly, 2012). Although previous discoveries have mainly been found from bacteria, it has been estimated that many yet undiscovered virus proteins will be of great use in the future applications (Song and Zhang, 2008; Keen, 2015).

1.2 Thermophilic bacteriophage P23-77

The phage P23-77 was initially discovered in samples collected from alkaline hot springs in New Zealand by the Promega Corporation (Yu et al., 2006). The phage is strictly lytic and infects the strain ATCC33923 of the gram-negative bacteria *Thermus thermophilus* (Yu et al., 2006; Jaatinen et al., 2008). P23-77 virions are icosahedral and they contain an internal lipid membrane underneath the capsid. The lipid membrane follows the shape of the capsid and encloses the circular 17 kb dsDNA genome. The genome contains 37 open reading frames, of which 10 are considered to code for structural proteins (Jalasvuori et al., 2009). The capsid and the internal lipid membrane are attached to each other via a yet unrecognized vertex complex at the five-fold vertices. The appearance of the P23-77 virion is spherical and it is 78 nm diameter in size (Jaatinen et al., 2008). The optimal temperature for P23-77 to infect *T. thermophilus* is approximately 70 °C. The phage is considerably thermostable and its infectivity is not affected during a storage period of 72 h at 28 °C. Thus, P23-77 is reasonably stable and easier to study in laboratory conditions than other similar thermophilic bacteriophages (Jaatinen et al., 2008).

1.3 Evolutionary links between P23-77 and other viruses

Initially, P23-77 was thought to belong to the viral family *Tectiviridae* due to its similarities considering the structure of the bacteriophage (Yu et al., 2006). P23-77 was compared to PRD1, which is the type species of the *Tectiviridae* (International Committee on the Taxonomy of Viruses, 2015). Both viruses have an icosahedral capsid and an internal lipid membrane, which encapsidates the dsDNA genome. In addition, they both have spikes on the capsid surface (Gowen et al, 2003; Abrescia et al., 2004; Jaatinen et al., 2008). However, further research revealed distinctive features in the structure of P23-77 compared to other viruses and virus families (Jaatinen et al., 2008, Jalasvuori et al., 2009, Rissanen et al., 2013). For instance, the capsid structure of P23-77 is more spherical than the capsid of PRD1 and it constitutes of two major capsid proteins (MCPs) instead of one (Bamford and Bamford, 1990; Jaatinen et al., 2008). P23-77 belongs to a new virus genus

Gammasphaerolipovirus of the novel family *Sphaerolipoviridae* (Pawlowski et al., 2014; ICTV, 2015). This novel family includes P23-77 along with other viruses infecting *Thermus* bacteria and halophilic archaea (Pawlowski et al., 2014). *Sphaerolipoviridae* is one of the four families, which spans two domains of life (ICTV, 2015).

The genomic analysis of P23-77 revealed it to be evolutionary related to SH1 and IN93, which represent *Haloarcula* and *Thermus* viruses, respectively. Accordingly, important genes, which are involved in the coding of the MCPs, are homologous between IN93, P23-77 and SH1 (Jalasvuori et al., 2009) SH1 belongs to the same virus family as P23-77, but to a different genus *Alphasphaerolipovirus* (Pawlowski et al., 2014). SH1 has the same T-number and shares the unusual capsid structure with P23-77 (Jaatinen et al., 2008; Jäälinoja et al., 2008; Jalasvuori et al., 2009) Yet, the capsid of P23-77 is more spherical and the spike structure is different from SH1 (Jäälinoja et al., 2008; Jaatinen et al., 2008). IN93 and P23-77 share the same family and genus (Pawlowski et al., 2014). Although P23-77 and IN93 share similarities in their genomic composition, IN93 possesses phage integrase, restriction endonuclease and prophage repressor genes in its integration cassette, which P23-77 lacks. Hence, P23-77 is strictly lytic while IN93 can enter the prophage state (Jalasvuori et al., 2009; Matsushita et al., 2009). Structurally P23-77 has been compared to the marine bacteriophage PM2 (Rissanen et al., 2013). P23-77 and PM2 both possess an internal lipid membrane, which encloses the circular dsDNA genome (Abrescia et al., 2008; Jaatinen et al., 2008). In addition, the β-barrels constituting the MCPs resemble each other in conformation (Rissanen et al., 2013).

The viruses IN93, SH1, PM2 and P23-77, share similarities with other tailless icosahedral viruses like the enterobacteriophage PRD1 in their packaging ATPase and the folding of their MCPs (Strömsten et al., 2005; Abrescia et al., 2008; Jäälinoja et al., 2008; Jalasvuori et al., 2009; Rissanen et al., 2013). The putative packaging ATPase is found from all membrane containing viruses of the PRD1 –adenovirus lineage (Strömsten et al., 2005; Jalasvuori et al., 2008). The PRD1- adenovirus lineage includes viruses, which infect hosts from different domains of life but share similarities in their structure (Benson et al., 1999; Krupovic and Bamford, 2008). For instance, adenovirus infects eukaryotic cells while SH1 and IN93 infect archaeal and bacterial cells, respectively (Porter et al., 2005; Krupovic and

Bamford, 2008; Matsushita et al., 2009). Due to the similarities between *Sphaerolipoviridae* and PRD1-adenovirus lineage, there might exist an evolutionary link between these viruses from different domains of life (Bamford et al., 2002; Strömsten et al., 2005; Maaty et al., 2006; Jalasvuori et al., 2009; Pawlowski et al., 2014).

1.4 The capsid structure of P23-77

The icosahedral capsid of P23-77 is formed from 270 hexameric and 12 pentameric capsomers and it has a triangulation (T) number of 28 (Jaatinen et al., 2008). The T number describes the quasi-symmetry of pentagons and hexagons in the icosahedral capsid shell (Caspar and Klug, 1962). The capsid is mainly build by two MCPs named virion protein (VP) 16 and VP17 (Jalasvuori et al., 2009; Rissanen et al., 2013). VP16 is a small MCP, approximately 20 kD in size while VP17 is a large MCP of 32 kD size. VP16 exists as a dimer and VP17 as a monomer. The MCPs form heterotrimers and heterotetramers with each other. Yet, some VP16 dimers are present in the capsid structure (Rissanen et al., 2013). At the virion vertices there are unidentified penton proteins to which narrow and straight spikes of approximately 15 nm length are attached (Jaatinen et al., 2008). According to the putative model created by Rissanen et al. (2013) the vertices from where the spikes protrude are surrounded by VP17, which is attached to VP16 dimer. The structure of P23-77 is depicted in Figure 1. Due to the unusual T number of 28 and capsid arrangement, the capsid structure of P23-77 is considered unique among phages (Jaatinen et al., 2008; Rissanen et al., 2013).

1. 5 The minor capsid protein VP11

P23-77 possesses a minor capsid protein VP11, which has been estimated to constitute the capsid together with the MCPs VP16 and VP17 (Jalasvuori et al., 2009; Rissanen, 2014). Dissociation experiments done to purified P23-77 virions show that VP16 and VP17 are released from the virion in 3 M urea or 3 M GuHCl conditions (Jalasvuori et al., 2009). In general, proteins are denatured in 3 M urea or 3 M GuHCl (Monera et al., 1994). By treating the virions with pH 6.0 VP11 was also released from the virion. The other proteins of P23-77 were associated with the aggregated DNA and the lipid membrane. Thus, VP16, VP17 and VP11 were concluded to be part of the external protein capsid of P23-77 (Jalasvuori et al., 2009).

The exact localization and function of the minor capsid protein VP11 is unknown. Yet, due to its dissociation behavior (Jalasvuori et al., 2009), VP11 might serve as a linker between the outer shell of the capsid and the internal lipid membrane. Although VP11 is not an integral membrane protein, it has been estimated to have similarities in function with the integral membrane proteins P3 and P6 of PM2 (Jalasvuori et al., 2009; Rissanen, 2014). These membrane proteins surround lipid vesicles in the internal lipid membrane and stabilize the mature PM2 virus capsid. In addition, they potentially aid in the capsid assembly of PM2 (Abrescia et al., 2008). In contrast, it has been suggested that VP11 could stay on the outer side of the capsid and localize at the vertices of the virus as the unidentified penton protein (Jaatinen et al., 2008). Supporting the theory, the 22 kD mass of VP11 is close to the mass of a monomeric penton protein (18 kD) (Jaatinen et al., 2008; Jalasvuori et al., 2009). No VP11 homologs have been found from other viruses, thus VP11 is considered as a unique protein (Jalasvuori et al., 2009; Pawlowski et al., 2014).

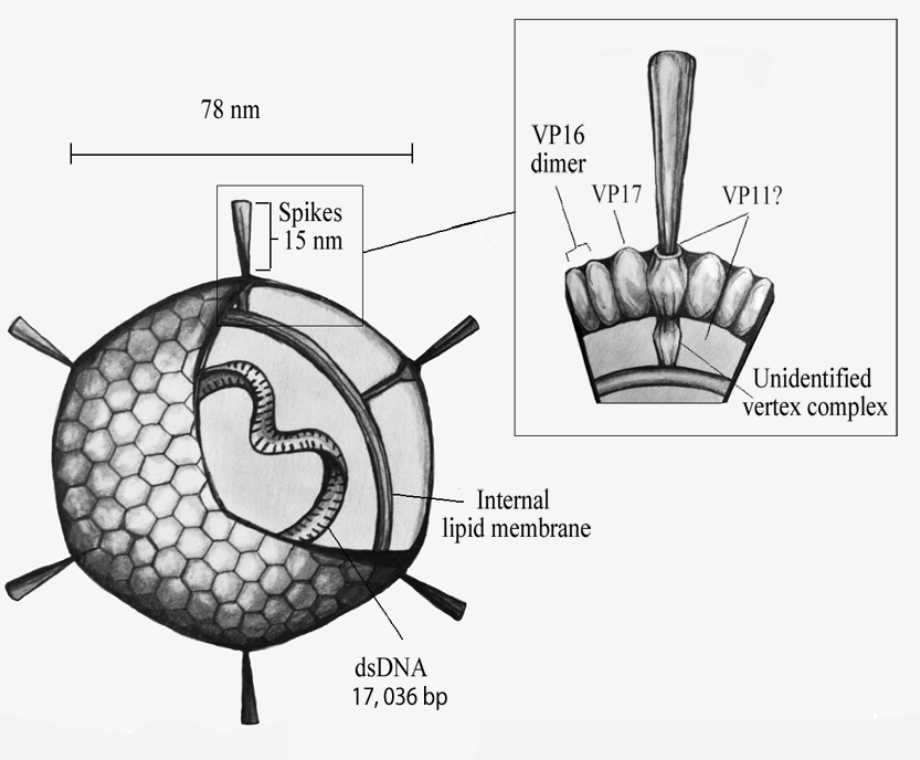

Figure 1. The structure of P23-77. The virion is 78 nm in size and has an internal lipid membrane enclosing dsDNA. There are spikes (15 nm) on the vertices of the capsid. On the right-hand side, there is a close-up image of the spike and vertex structure. A model of the vertex structure created by Rissanen et al. (2013) suggests that VP16 dimers and other oligomers are attached to VP17 trimers, which surrounds the vertices. VP11 is hypothesized to localize between the internal lipid membrane and the capsid shell or at the capsid vertices. Modified from Jaatinen et al. (2008).

2. Aims of the Study

The main focus of the study was to research the location of VP11 in the capsid of the bacteriophage P23-77. Without the knowledge of the location of the minor capsid protein VP11, the capsid structure for P23-77 cannot be constructed. In addition, the location gives information about the function of the protein. The hypothesis was that VP11 is located between the internal lipid membrane and the capsid shell or on the outer side of the capsid as a penton protein. The experiments contained optimization of a western blot method for VP11 detection as well as testing the functionality of the anti-VP11 serum. The copy number of VP11 was estimated and its location was studied visually with transmission electron microscopy and immunolabelling.

The concrete aims of the study were to:

1. Optimize a western blot method for the detection of VP11
2. Estimate the copy number of VP11 in the P23-77 capsid
3. Study the localization of VP11 visually with transmission electron microscopy and immunolabeling

3. Material and Methods

3.1 Bacterial Strains and Viruses

Thermus thermophilus strain 33923 from the American Type Culture Collection (ATCC33923, Manassas, VA) was used as a host strain for the cultivation of the bacteriophage P23-77. Bacterial agar plates were cultivated in a humid atmosphere to prevent the medium from drying out. The plates were placed into a sealed plastic box containing water on the bottom on a stack of empty pipette trays to prevent the plates from touching the water. *T. thermophilus* was cultivated at 70 °C in *Thermus* medium (TM), supplemented with either polypeptone for liquid cultures or peptone for agar plates. The media were prepared according to the instructions in Appendix 1.

Enterophage PRD1 was propagated by using *Salmonella enterica* DS88 as a host strain. The strain contained the plasmid pLM2, which makes the cells susceptible to PRD1 infection (Bamford and Bamford, 1990). Incubation of *S. enterica* was performed at 37 °C in Luria Bertani (LB) -medium containing 50 µg/ml kanamycin. PRD1 served as a control for the performed transmission electron microscopy (TEM) experiments.

3.2 Plaque assay

The plaque assay was used for estimating the virus titer, the concentration of infectious virus particles in a sample. The protocol was initiated by preparing a liquid culture for *T. thermophilus* by inoculating a few bacterial colonies in 20 ml of fresh media. The culture was then incubated at 200 rpm and 70 °C for 4-6 h. Usually 100 µl of appropriate diluted P23-77 virus sample were mixed with 200 µl of the cultivated host strain in 3 ml of TM soft-agar and poured on TM plates. The plates were incubated at 70 °C for 16 h. Plaques arising from each infectious particle were counted and the quantity of virus was estimated as plaque forming units per millilitre (pfu/ml). In this study, the P23-77 virus concentration was always conducted according to the method described above. For the estimation of the PRD1 titer, the plaque assay protocol was performed as described above with a few exceptions: the host *S. enterica* was incubated at 37 °C overnight and liquid cultures were made in LB media.

3.3 Propagation of *Thermus* phage P23-77

3.3.1 Preparation of virus stocks

In order to gain high amounts of viral material for large scale purification, a fresh virus stock of P23-77 had to be produced. For this purpose, the host *T. thermophilus* ATCC33923, either freshly taken from -80 °C glycerol stock or -20 °C stock, was plated and incubated at 70 °C for 16 h. Cells were re-streaked three times on a new TM plate to make the host more susceptible for the P23-77 infection. For a new virus stock, confluent lysed soft-agar plates were prepared by using a previously made P23-77 virus stock. An appropriate amount of virus solution was mixed with 3 ml of soft-agar and 200 µl of host strain and plated on to TM-agar plates. Generally, confluent P23-77 soft-agar plates were received with 100 µl of a 10^{-4} viral dilution and 200 µl of a 10^{-5} viral dilution. Plates were incubated at 70 °C for 6 h. The soft-agar layers containing the virus were harvested and diluted in TM-liquid medium (4 ml/plate). The solution was incubated at 150 rpm and 28 °C for 4 h followed by centrifugation at 12 000 rpm and 25 °C for 20 min (Sorvall Instruments RC5C, rotor SLA3000). The supernatant containing P23-77 was stored at room temperature. The freshly prepared virus stock was titered to estimate its infectivity by using the plaque assay technique.

3.3.2 One step Growth

A liquid culture of the host strain *T. thermophilus* was made from a freshly streaked bacterial plate by inoculating a few bacterial colonies to 200 ml of *Thermus* media. The culture was incubated at 200 rpm and 70 °C for 24 h. A spectrophotometer (Uvmini-1240 UV-VIS, Shimadzu) was used to measure the optical density at wavelength 550 nm (OD550) of the overnight culture. The overnight culture was diluted in fresh *Thermus* media to obtain an OD550 value of 0.4 for a new liquid culture. The fresh liquid culture was incubated at 200 rpm and 70 °C for approximately 3-4 h to an OD550 value of 1.3. The OD550 value 1.3 corresponds to 7×10^8 colony forming units (cfu)/ml (Jaatinen et al., 2008). At this point, the *T. thermophilus* culture was infected with a fresh P23-77 virus stock by using a multiplicity of infection (MOI) of 20. The OD550 value dropped to 0.5 after the infection had taken place, approximately 2.5 h later. The lysate that formed during the infection was centrifuged at 7000 rpm and 25 °C for 20 min (Sorvall Instruments RC5C, SLA 3000-rotor). The supernatant containing P23-77 was collected for virus purification.

3.4 Purification of P23-77 virus particles

In order to purify P23-77, the virus had first to be precipitated from the lysate that formed during the one step growth procedure. The precipitation was performed by adding 12 % polyethylene glycol (PEG) 6000 and 0.5 M NaCl to the lysate supernatant (see 3.3.2) and incubating the mixture at 180 rpm and 28 °C for 35 min. The precipitation was followed by centrifugation at 7000 rpm and 25 °C for 20 min to pellet the virus (Sorvall Instruments RC5, rotor SLA 3000). The virus pellet was rinsed with TV-buffer (see Appendix 1) and suspended in 1/15 of its original volume. The P23-77 precipitate was further purified by linear sucrose gradient centrifugation. Due to the fact that the virus aggregates during storage, the virus sample was centrifuged at 7000 rpm and 25 °C for 5 min prior to the sucrose gradient purification (Sorval Instruments RC5, rotor SS34). A gradient master device was used to prepare a 5-20 % (w/v) linear sucrose gradient in TV-buffer with the following settings: short, for SW28 tubes, 5-20 % and duration of 1 min 40 s (Biocomp). When the gradient was ready, 2.2 ml of gradient liquid from the top was replaced with the same amount of P23-77 virus. The gradient tubes containing P23-77 were applied to rate zonal centrifugation at 23 000 rpm and 25 °C for 45 min (Beckman Coulter ultracentrifuge, rotor SW28). The ultracentrifugation was followed by the collection of viral particles from the light scattering zone that formed during the centrifugation. The light scattering zone was visible under Visilight-VWR I-LED light. The purification by rate zonal centrifugation was followed by a second purification step using equilibrium centrifugation (2x-purification) or by pelleting the phage by centrifugation at 32 000 rpm and 25 °C for 2.5 h (Beckman Coulter centrifuge, rotor 45Ti) (1x-purification).

2x-purification of virus samples was performed by equilibrium centrifugation in 1.3 mg/ml cesium chloride in TV-buffer. First, 26 ml of cesium chloride was pipetted in SW-28 tubes and the tubes were balanced. Next, 10.5 ml of 1x-purified virus was applied on top of the tubes and balanced carefully. After balancing, samples were centrifuged at 21 000 rpm and 25 °C for 16 h (Beckman Coulter ultracentrifuge, rotor SW28). Ultracentrifugation was followed by the collection of viral particles. The virus particles were visible under Visilight-VWR I-LED light as three separate light scattering zones. The uppermost and most intensive light scattering zone was collected. The virus band was diluted 1:3 in TV-buffer and collected by differential centrifugation at 32 000 rpm and 25 °C for 2.5 h (Beckman Coulter ultracentrifuge, rotor 45 Ti). After centrifugation, the supernatant was

discarded: the tubes were rinsed with TV-buffer and carefully dried with Kleenex. The viral pellet was suspended in TV-buffer and stored at room temperature. Virus titers were estimated for each purification step to follow the changes in the infectivity of the virus. The Bradford method was used to measure the protein concentration of purified virus samples (Bradford, 1976).

3.5 Propagation and purification of bacteriophage PRD1

Bacteriophage PRD1 was propagated and purified by 1x-purification to be used as a control sample for P23-77 in TEM studies. The one step growth and 1x-purification of PRD1 phage was done in a similar manner as for P23-77 with a few exceptions. First, semi-confluent LB plates were prepared for the PRD1 stock preparation instead of fully-confluent plates. For semi-confluent PRD1 soft-agar plates, 250 µl of a10^{-9} viral dilution or 25-50 µl of a 10^{-8} dilution was used. The plates were incubated at 37 °C overnight. The soft-agar layer containing the virus particles was harvested as described for P23-77 (see 3.3.1) but the incubation was performed at 200 rpm and 37 °C for 3 h, followed by centrifugation at 10 000 rpm and 4 °C for 10 min (Sorvall Instruments RC5, rotor SS34). The PRD1 supernatant was filtered with a 32 mm syringe filter and stored at 4 °C.

In the one step growth propagation of PRD1, the liquid culture of the host *S. enterica* was incubated at 200 rpm and 37 °C for 24 h. The overnight liquid culture was first diluted to 1:8 in LB-medium before the OD550 value, which ought to be 0.34-0.6, was measured. The culture was incubated at 37 °C and 200 rpm until the OD550 reached the value 1.1. At this point, the liquid culture was infected with PRD1 with a MOI of 7. The culture was incubated at 37 °C and 200 rpm until the OD550 value dropped to 0.4, approximately 2 h later. The bacterial lysate was centrifuged at 8000 rpm and 4 °C for 15 min (Sorvall Instruments RC5, rotor SLA-3000).

For the precipitation of PRD1, 10 % PEG 6000 and 0.5 M NaCl were utilized. Dissolving was performed by magnetic stirring at 4 °C for 30 min. The precipitate was collected by centrifugation at 8000 rpm and 4 °C for 20 min (Sorvall Instruments RC5, rotor SLA 3000). The obtained virus sample was suspended in 20 mM of kalium-phosphate (KPO_4) pH 7,2 buffer and stored at 4 °C. PRD1 was centrifuged at 9000 rpm and 4 °C for 15 min to remove aggregates. For the 5-20 % sucrose rate zonal centrifugation, solutions were prepared in 20 mM KPO_4 –buffer instead of TV buffer. The rate zonal centrifugation was

performed at 24 000 rpm and 15 °C for 1 h (Beckman Coulter ultracentrifuge, rotor SW28). After collection of viral particles, PRD1 was pelleted by centrifugation at 33 000 rpm and 5 ° C for 3 h (Beckman Coulter ultracentrifuge, rotor SW28). The virus pellet was suspended in KPO_4 buffer and stored at 4 °C.

3.6 Transmission Electron Microscopy (TEM)

All grids that were utilized in the TEM experiments were treated as follows: For 200-mesh copper grids support films of 0.7 % Butvar (Electron Microscopy Sciences) in chloroform were made in University of Oulu and University of Jyväskylä. Grids were treated with Glow Discharge treatment to make the surface of the grids hydrophilic (EMS/SC7620 Mini Sputter Coater). Hydrophilicity aids the attachment of the virions to the grid. The settings used for the glow discharge were as follow: glow discharge 15 sec, 30 mA, 6×10^{-2} mbar.

3.6.1 Negative Staining

Negative staining was initiated by attaching the 1x- or 2x-purified virus to the grid. The attachment was performed by facing the grid down on a 20 µg/ml virus drop and incubating for 30 s. Excess liquid was removed from the grid by touching the edge with Whatman filter paper. The grid was placed on top of the negative stain solution for 15-20 s. The virus particles were stained either with 1 % or 2 % phosphotungstate acid (PTA) or 1 % ammoniumolybdate. All stains were used in neutral pH. Excess liquid was removed and the grids were left to dry in a grid box at least for 10 min before imaging them at JEM-1400 TEM (JEOL) with 80 kV and multiple magnifications and locations on the grid.

3.6.2. Immunolabeling of VP11

The immunolabeling of the virions (1x- and 2x-purified) and viral proteins were done in a quiet location to reduce the possibility of contamination of the samples. Due to the high magnification used at TEM, even minute impurities may appear on the sample and disturb the visualization of the particles being studied. Immunolabeling steps were performed on a parafilm attached to a table with water. The parafilm base was covered with a glass petri dish to create a humid atmosphere for the immunolabeling. All solutions used for the immunolabeling, except 10 % fetal calf serum (FCS) and antibodies, were filtered with a syringe filter of 32 mm containing polyethersulfone Supor® membrane 0.8/0.2 µm (Life Sciences). 10 % FCS was filtered by centrifugation at 12 000 g for 5 min (Eppendorf).

Grids were treated with glow discharge (see above). The Labelling was done by the drop application method, in which the grid is transferred with forceps from droplet (15-20 µl) to droplet containing different substances. Each sample was done in duplicate or triplicate. The protocol for immunolabeling of VP11 was modified from the immunolabeling protocol of PRD1 (Gowen et al., 2003).

The immunolabelling was initiated by attaching the virus (in concentration 20 µg/ml) or pure VP11 protein (in concentration 100 µg/ml) to the grid. This step was performed in a similar manner as in the negative staining procedure (see 3.6.1) with the exception that the grid was incubated for 1 min on the sample. Next, the sample was treated with 0.5 % glutaraldehyde (Electron Microscopy Sciences) in phosphate buffered saline (PBS) for 15 min to preserve the structure of the virus or protein. The glutaraldehyde treatment was followed by three washes with PBS by incubating the grid on three separate PBS drops for 5 min. The grid was then treated with 0.02 % glycine in PBS for 15 min for glutaraldehyde neutralization. Washing steps were repeated as described above with PBS containing 0.02 % glycine to remove the traces of glutaraldehyde. The grid was then placed on 10 % FCS in PBS for 10 min, which was followed by labelling the sample with polyclonal primary antibody anti-VP11 serum (Storkbio) produced in two different rabbits (193, 194) for 30 min. The anti-VP11 serum (194) was diluted 1:10 (concentration not mentioned by the producer) in PBS containing 10 % FCS. Preceding the primary antibody treatment, excess liquid was removed from the grid by touching the edge of the grid with Whatman filter paper. The drying step was done to avoid diluting the antibody concentration on the drop. For pure VP11, primary antibody dilutions of 1:100 and 1:1000 were tested in order to study the sensitivity and specificity of the primary antibody.

After labelling with primary antibody, the grid was washed three times in PBS containing 10 % FCS as described above. 10 nm Protein A gold (cmc-utrecht) was used as the secondary antibody to detect the anti-VP11 serum. Protein A gold was used in dilution of 1:50 (concentration not mentioned by the producer) in 10 % FCS in PBS. Grids were incubated 30 min in the protein A gold label and then washed four times with PBS, each wash for 5 minutes. Afterwards the grids were washed three times with sterile water, each for one minute. Negative staining was done as described above (see 3.6.1) with ammoniumolybdate or 1 % PTA. The prepared grids were imaged at JEM-1400 TEM

(JEOL) with magnifications from 6000 x to 100 000 x, with voltage 80 V at least from three different locations on the grid.

3.6.3 Controls for Immunolabeling

PRD1 was used as a positive control to test whether the immunolabeling protocol was performed successfully. The immunolabelling protocol was performed in the same manner as for P23-77 but with different primary antibody. The P2 vertex protein was labelled with anti-P2 serum diluted 1:20 in PBS containing 10 % FCS. As a negative control, pre-immune serum of VP11 (194) was used instead of anti-VP11 serum (194) to ensure that the serum was not recognized by the protein A-gold particles.

3.7 Western Blotting and Immunodetection of VP11

3.7.1 SDS-PAGE

15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for mini (8.5 cm x 4.5 cm) and large gels (16 cm x 14 cm) for Western blot detection (Olkkonen and Bamford, 1989). Run settings for minigels were as follows: 150 V, 180 mA with run time of 1 h 45 min. Large gels were first run 80 V, 20 mA for 16 h, followed by a run of 200 V, 187 mA for 2 h. Precision Plus Protein^{TM} All Blue Standards (Biorad) was used as a molecular ladder. Preceding the gel run, virus samples were boiled for 10 min in reducing loading buffer containing either 1 % or 5 % β-mercaptoethanol. Purified virus material (2x) was applied in concentrations of 2.2-6.6 μg per lane in minigels. For large gels the applied amount varied from 36 to 72 μg per lane.

3.7.2 Western blot

Western blot was performed with a three buffer system by using the TE-77 Enhanced Chemiluminescence semi-Dry Transfer Unit with conditions of 180 mA (4.0 mA/cm^2), 200 V and 25 min run time (Amersham Biosciences, EMD Millipore). In addition, wet tank blotting was tested with conditions 400 mA, 100 V and run time 1 h. In order to study the transfer of VP11 from SDS-PAGE to Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Millipore), SDS-PAGE was run with duplicate protein samples. After the run, the SDS-PAGE was cut in half. One half of the gel was stained with Coomassie blue after the run, the other after western blot. Protein transfer was verified by staining the PVDF membrane with Ponceau S for 15 min at room temperature (Hassan J. et al., 1987).

3.7.3 Immunolabeling

The SNAP i.d. 2.0. Protein Detection System (EMD Millipore) was applied for the immunodetection of VP11 on the PVDF membrane. The immunolabeling was done according to the manufactorer´s instructions (EMD Millipore) with the exception that blocking of the PVDF membrane was done overnight (24 h) or over the weekend (72 h). For the blocking, 1xTEN buffer (50 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl) containing 1 % Tween 20 was used. Blocking was performed on a rocker at 4 °C. VP11 was labeled with polyclonal primary antibody anti-VP11 serum produced either in rabbit 193 or 194 (Storkbio). The thawed anti-VP11 antibody was treated with 0.05 % sodium azide for storage at 4 °C. After the primary antibody labelling the secondary antibody polyclonal swine anti-rabbit immunoglobulin conjugated with horseradish peroxidase (HRP, Dako Cytomation), was introduced to the PVDF membrane. Washes with 1xTEN buffer containing 0.01 % Tween 20 were made in between the primary and secondary antibody immunolabellings. Signals were received after incubating the PVDF membrane in freshly prepared detection liquid of Enhanced Chemiluminescence –Kit for 5 min (Thermo Scientific). Best signals were received when the primary antibody was used in dilution of 1:30 000 (stock concentration not mentioned by the producer) and secondary antibody in concentration of 0.26 μg/ml, both incubated for 10 min. The developed PVDF membrane was imaged with Quantity One program by using Chemi Hi Sensitivity light (Biorad). During the experiments, the stability of anti-VP11 serum was noticed to diminish after two months storage in 4 °C. Detection limit of Western blot was analysed with recombinant VP11 chromatographically purified from *Escherichia coli*. Protein samples from 1 μg- 50 pg were applied to a mini SDS-PAGE gel (8.5 cm x 4.5 cm) and run as described above.

3.8 Estimation of the VP11 Capsid Copy Number

The copy number of VP11 was estimated from large 15 % SDS-PAGE and tricine-SDS-PAGE gels (Schagger and Jagow, 1987). For the estimation Quantity One program (Biorad) was used according to the manufacturer's instructions. The program detects each protein band on the gel individually and calculates their relative intensity from 0-100 %. VP16 was used as a reference band and its measured intensity was stated as 1080 copies of protein based on the amount of VP16 copies in the capsid (Rissanen et al., 2013). The measured intensities of VP16, VP17 and VP11 were calibrated with the molecular masses of the proteins. The copy numbers for VP11 and VP17 were obtained by dividing their

calibrated intensities with the calibrated intensity of VP16 and multiplying the result by 1080. Disc value for background reduction was 10, width of lanes 30 mm.

4. Results

4.1 Production of highly purified infective virus particles

In order to gain good concentration of highly purified virus for TEM and western blot studies, P23-77 was propagated and purified by using rate zonal centrifugation with sucrose gradient (1x-purification) and equilibrium centrifugation in cesium chloride and differential centrifugation (2x-purification). Titers and the sample volumes were estimated after each purification step to learn the infectivity changes of the virus sample during the protocol. For the lysed virus sample the average absolute infectivity was 4.7×10^{13} pfu, for the PEG precipitate 1.9×10^{13} pfu, for the rate zonal centrifugation gradient band 5.4×10^{12} pfu and for the equilibrium centrifugation gradient band 4.6×10^{12} pfu. The differential centrifugation virus sample had the lowest absolute infectivity with the value of 8.0×10^{11} pfu (Table 1). The average specific infectivity value was determined only for the final sample (differential centrifugation), which was 1.32×10^{12} pfu/mg. There was a hundred-fold change in the absolute infectivity of the initial sample (the lysate) and the final sample (differential centrifugation) (Table 1). Hence, the infectivity of the virus sample decreased during the purification. The loss of infectivity was calculated as percentages for each virus sample compared to the lysed virus sample. The total loss of infectivity from the beginning of the purification process (lysate) to the final sample (differential centrifugation) was 98 % (Table 1). Additionally, there was a loss of sample volume during the process. Accordingly, from 980 ml viral culture only a yield of 250 µl was obtained. Yet, the virus sample contained enough infective viruses for following studies (3.2×10^{12}, Table 1). It was noticed that the virus stock was stable for two weeks in PBS and TV-buffers (no drop in titers). Bradford protein concentration was measured after each differential purification procedure to estimate the needed virus amount for TEM immunolabeling studies. Bradford protein concentration values for purified P23-77 ranged from 1.0 - 2.25 mg/ml (Bradford, 1976). Hence, there was some variation of protein concentration in each purification procedure.

Table 1. P23-77 infectivity and sample volume changes during the virus purification process

	Lysate	PEG precipitate	Rate zonal centrifugation gradient band	Equilibrium centrifugation gradient band	Differential centrifugation
Average pfu/ml	4.8×10^{10}	2.8×10^{11}	3.6×10^{10}	1.1×10^{11}	3.2×10^{12}
Volume (ml)	980	66	150	42	0.250
Absolute infectivity (pfu)	4.7×10^{13}	1.9×10^{13}	5.4×10^{12}	4.6×10^{12}	8.0×10^{11}
Loss of infectivity	0 %	60 %	89 %	90 %	98 %

4.2 Negatively stained and immunolabelled P23-77 visualized with TEM

4.2.1. Negative staining of P23-77 successful

P23-77 virions (2x-purified) were stained with different negative stains to study how the virus reacts with them. First 30 s dyeing time was used for the negative staining but it resulted in too heavily stained samples. Thus, the incubation time was decreased to 15-20 s. 1 % Ammoniumolybdate and 1 % and 2 % PTA labels successfully stained P23-77 (Figure 2a). There was no difference noticed in the staining results between the different negative stains. The negative staining was performed after the purification process of P23-77. Thus, the staining results showed that P23-77 was not disrupted during the virus purification process. Accordingly, spherical viral particles filled with DNA (visible as white spots) were seen with TEM (Figure 2a). Some virus samples were stained after two weeks of the virus purification. The morphology of the P23-77 virion was noticed to remain the same after two weeks of the virus purification, indicating a high stability of the virus particles under the chosen storage conditions (TV-buffer, RT).

4.2.2. Immunolabelling experiments of P23-77 and PRD1

In order to study the location of VP11 in the P23-77 virion, P23-77 was immunolabelled and visualized by using TEM. Purified viruses (either 1x or 2x) were immunolabeled with anti-VP11 serum (194) and Protein A gold (10 nm). The morphology of the 1x-purified viruses was noticed to be better than 2x-purified viruses. Viruses purified by 1x method were spherical and filled with DNA, whereas the 2x-purified viruses were clumped together and had lost their spherical appearance. Possibly, the remnants of chemicals from the 2x-purification interfered with the reagents of the immunolabelling and caused the rupture of P23-77. There was no attachment of protein A-gold labels with the purified P23-77 virions in the immunolabelling experiments (Figure 2b). Thus, it can be suggested that

VP11 is located inside the virion, not exposed on the outer shell. Yet, the immunolabelling protocol for TEM is not fully optimized and it might have had an impact on the results.

In order to verify that the immunolabeling protocol was performed correctly, PRD1 was immunolabeled by using the same protocol as used for P23-77. Anti-P2 serum was used as the primary antibody, which attaches to the P2-vertex structures in the PRD1 virion (Gowen et al., 2003). All other reagents utilized in the labelling of PRD1 were same as for P23-77. There was a specific binding of the secondary label (protein A gold) to the P2-vertices on the PRD1 virions after the immunolabelling (Figure 2c). Thus, it can be deduced that the secondary label and the immunolabelling protocol were working correctly. The PRD1 virions were more hexagonal than P23-77 virions and they possessed hollow tubes (Figure 2c.), which are used to transfer the viral genome during infection (Gowen et al., 2003). Similar kind of hollow tubes were not seen in P23-77 viral particles.

A set of studies was conducted to examine the quality of the anti-VP11 serum. To assess the effectiveness, immunolabeling was performed for pure VP11 protein (100 µg/ml). When compared to the immunolabelled P23-77 virions, a higher concentration of protein A Gold was present on pure VP11 than on samples containing P23-77. The result indicates that the anti-VP11 serum was labeling VP11 effectively. In the comparison both samples were treated with anti-VP11 serum diluted in 1:10 in PBS. In order to determine the detection limit of the anti-VP11 serum, a dilution series was made from 1:10 to 1:1000. There was no difference in the amount of protein A-gold particles on the grids between the different dilutions (data not shown). Hence, the specificity limit of anti-VP11 serum was not determined. In addition, 2x-purified virus sample and VP11 protein were labelled with the pre-immune serum of VP11 (194) instead of the anti-VP11 serum to test the specificity of the secondary label and to see the amount of background labels. The samples treated with serum showed only minute amounts of protein A-gold particles. Due to the low number, the secondary labels seen were deduced as background labels. According to the results, the serum was not recognized by the protein A gold.

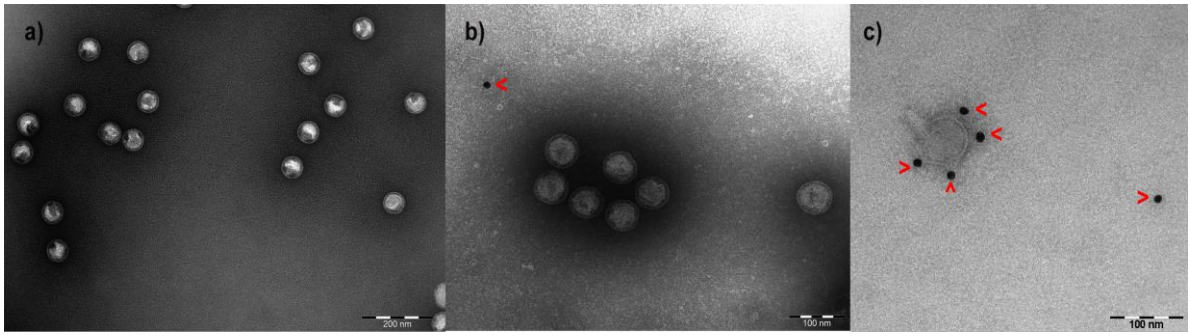

Figure 2. Negatively stained and immunolabelled P23-77 and PRD1 virions. Protein A-gold particles are visible in the images as small black dots marked with red arrows. a) 2x-purified P23-77 stained with 1 % ammoniummolybdate. Virions are spherical and filled with DNA. Scalebar stands for 200 nm. b) 1x-purified P23-77 immunolabelled with anti-VP11 serum and protein A gold (10 nm). Virions were negatively stained with 1% ammoniummolybdate. Scalebar stands for 100 nm. c) PRD1 vertex protein P2 immunolabeled with anti-P2 serum and protein A-gold (10 nm). Scalebar stands for 100 nm.

4.3 The Immunodetection of VP11

In order to optimize a Western blot method for the detection of VP11, purified virus (1x and 2x) material was immunolabeled with anti-VP11 serum and horseradish peroxidase (HRP) with varying conditions. The aim was to receive a strong and specific signal of VP11 without background or unspecific labels. First, the conditions recommended by the SNAP i.d. 2.0. Protein Detection System were tested (EMD Millipore). After many trials, the optimal conditions for immunodetection of VP11 were found. The different conditions used for the optimization process can be seen in Table 2. Conditions 1-4 produced blots with a lot of background labels, whereas conditions 5-7 did not result in specific signal. The optimal conditions did not produce any background labels or unspecific signals.

The anti-VP11 serum was produced in two different rabbits (193, 194) against the native VP11 protein. Both antibodies were tested in the immunodetection experiments. The anti-VP11 serum produced in the rabbit 194 was noticed to be more sensitive than 193 in the detection of VP11. The best detection was received by using anti-VP11 serum 194 in dilution 1:30 000 (stock concentration not informed by the producer) and secondary antibody (HRP) in concentration of 0.26 µg/ml. Incubation time used for both antibodies was 10 minutes (Table 2). The specific signal received for VP11 using the optimal immunodetection conditions can be seen in Figure 3. Higher virion protein concentration on SDS-PAGE gave better signals when raised from approximately 2 µg to 4 µg on minigels (8.5 cm x 4.5 cm). For large gels (16 cm x 14 cm), the western blot signal was

enhanced when the protein concentration was raised from 30 μg to 40 μg. Further increase in protein concentration did not enhance the signal in either gel system.

Table 2. Optimization and optimal conditions for VP11 immunodetection

Trial	Anti-VP11		HRP	
	Dilution	**Incubation time**	**Concentration**	**Incubation time**
1	1:2000	10 min	0.43 mg/ml	10 min
2	1:10 000	10 min	0.43 mg/ml	10 min
3	1:10 000	60 min	0.13 mg/ml	10 min
4	1:20 000	10 min	0.13 mg/ml	60 min
5	1:40 000	10 min	0.13 mg/ml	60 min
6	1:30 000	10 min	0.13 mg/ml	60 min
7	1:10 000	10 min	0.26 mg/ml	10 min
Optimal conditions	**1:30 000**	**10 min**	**0.26 mg/ml**	**10 min**

The detection limit of the VP11 antibody was analyzed with recombinant VP11 chromatographically purified from *E. coli*. Protein samples in concentrations ranging from 50 pg to 1 μg were fully reduced with sample buffer containing 5 % β-mercaptoethanol and applied to a 15 % SDS-PAGE minigel. The antibody was able to detect VP11 in concentrations from 1 μg to 50 ng (data not shown). Thus, the detection limit of the anti-VP11 serum (194) was 50 ng. The transfer of different virus proteins varies a lot. VP11 is mainly retained in the SDS-PAGE as seen in Figure 3a. Remaining VP11 in the SDS-PAGE may reduce the defined detection limit of the primary antibody.

To estimate the effectiveness of protein transfer from the 15 % SDS-PAGE gel to the PVDF membrane, a large gel was cut in half and stained with Coomassie blue before and after western blot. Protein bands were well stained in the gel dyed before western blotting. Protein bands were clearly visible on the dyed blotted gel as well, demonstrating an incomplete transfer of protein to the membrane (Figure 3a). Hence, wet tank blotting was performed to see if there was an improvement of protein transfer. Tank blotting of VP11 did not give better results than the semi-dry system and thus, the experiments were continued with the semi-dry system.

To assess if VP11 exists as a dimer structure in the virion, the virus samples were treated with reducing agent in two different concentrations. Virus particles received from 2x-purification were treated with SDS-loading buffer containing either 1 % or 5 % β-mercaptoethanol. Earlier studies with purified VP11 had shown the existence of 50 kD

protein band on SDS-PAGE when using 1 % β-mercaptoethanol. When sample buffer containing 5 % β-mercaptoethanol was used, only 25 kD protein band was detected. It has been shown previously that proteins forming strong disulfide bridges may not be totally reduced by boiling them in a sample buffer containing 1 % β-mercaptoethanol (Grigorian et al.2005). By using a higher concentration (5 %) of the reducing agent, it was expected that all the multimers of VP11 would be reduced to their monomeric forms due to the disruption of the disulfide bridges between the proteins. Protein samples treated with 1 % β-mercaptoethanol showed two distinctive bands (25 kD and 50 kD) on the gel and blot (Figure 3b), whereas samples containing 5 % β-mercaptoethanol showed only one band 25 kD in size (Figure 3c). Accordingly, results suggest that VP11 exist as a dimer in the virion and is reduced to its monomeric form when treated with higher concentration of the reducing agent β-mercaptoethanol. Moreover, no larger than 50 kD multimers were seen on the blots or the SDS-PAGE gels, which supports the idea that VP11 exists as a dimer. The transfer of VP11 was not complete in the western blot. The intensity of VP11 band was faintly diminished in the gel stained after western blot indicating a weak transfer of protein. In contrast, the intensities of the bands of the MCPs are clearly decreased, indicating a good transfer of protein. In the immunolabelled blot only VP11 was detected (Figure 3a).

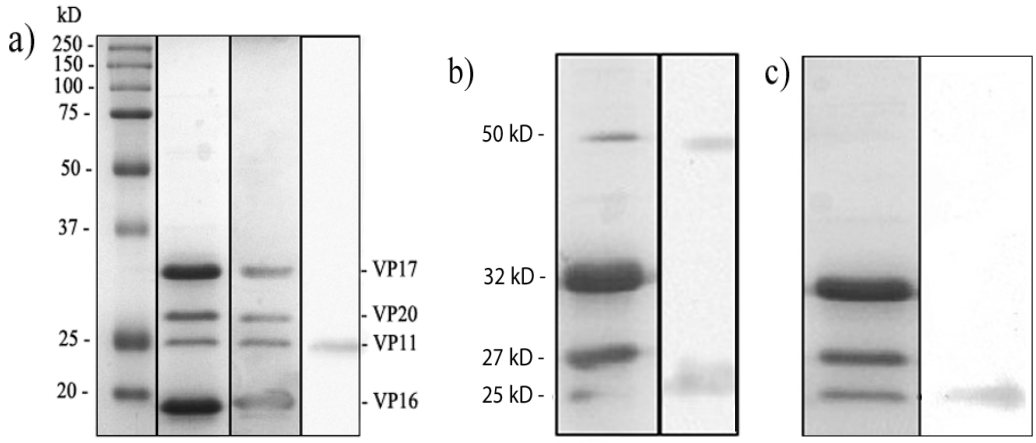

Figure 3. a) Transfer of VP11 from 15 % SDS-PAGE gel to a PVDF membrane. The gel was stained with Coomassie blue before western blot (left-hand side) and after (in the middle). Virus proteins 17, 20, 11 and 16 are showing on the stained gels. Immunolabelled PVDF membrane (right-hand side) shows specific detection of VP11. Virus sample was treated with 5 % of the reducing agent. b) Virus sample treated with 1 % β-mercaptoethanol showed two protein bands for VP11. Monomer (25 kD) and dimer (50 kD) bands showing on Coomassie blue stained gel (left-hand side) and PVDF immunolabelled membrane (right-hand side). In between, there are VP20 (27 kD) and VP17 (32 kD) bands. c) Virus sample treated with 5 % β-mercaptoethanol showed only 25 kD monomer band for VP11. Stained gel is on the left-hand side and the

immunoblotted membrane on the right-hand side. Protein ladder used was Precision Plus Protein™ All Blue Standards (Biorad).

4.4 VP11 Copy Number

In order to receive more information of the function of VP11, its copy number in the P23-77 capsid was estimated. Protein copy number gives an idea how the protein is organized in the virion. Copy number of VP11 was estimated with Quantity One program (Biorad) from large 15 % SDS-PAGE gels. The program was used to detect the bands on the gel and measure their relative intensities. An estimation performed for a 15 % SDS-PAGE gel can be seen in Figure 4. Relating to the measured intensities the copy numbers of the capsid proteins, VP11, VP16 and VP17 could be calculated (see 3.8). It was assumed that Coomassie stain, which was used to dye the gels, stains the proteins on the gel equally. Estimated copy number for VP17 was 527, which is close to the theoretical value 540 (Rissanen et al., 2013). Copy number of VP11 was estimated to be 147. The most abundant P23-77 protein VP16 served as the reference band (1080 copies per capsid).

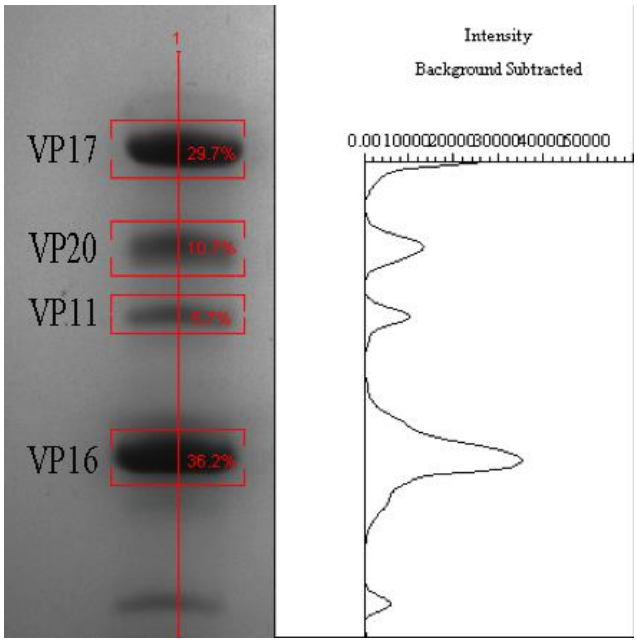

Figure 4. VP11 copy number estimation from 15 % SDS-PAGE gel. Samples were treated with 5 % β-mercaptoethanol to reduce VP11 to its monomeric form. Quantity one (Biorad) program was used to detect the protein bands on the gel and calculate their relative intensities. For VP16 the intensity was 36.2 %, for VP17 29.7 %, for VP20 10.7 % and for VP11 5.7 %. Next to the detected protein bands, there is a plot depicting the intensities of the proteins after subtracting the background of the gel.

5. Discussion

P23-77 is a quite recently discovered thermophilic phage (Yu et al., 2006) and consequently, it has not been studied thoroughly. For instance, the exact capsid structure of P23-77 has not been established. Previous studies have shown that three proteins, VP16, VP17 and VP11 are associated with the capsid (Jalasvuori et al., 2009; Rissanen et al., 2013). Locations of the MCPs have been revealed (Rissanen et al., 2013) but the function of the minor capsid protein VP11 remains unsolved. The aim of this thesis was to study the location of the minor capsid protein VP11 and to optimize a western blot method for VP11 detection. Resolving the location of VP11 would help to construct the whole capsid structure for P23-77. The capsid structure of P23-77 is considered unique (Jaatinen et al., 2008) and is in itself fascinating to study. Additionally, studying the structural components of viruses may reveal evolutionary relationships, because structural components remain rather preserved over time (Bamford et al., 2002; Maaty et al., 2006). For instance, the PRD1-adenovirus lineage includes viruses, which infect *Bacteria*, *Archaea* and *Eukaryota*. These viruses are grouped in the same lineage due to their similarities in structure. Accordingly, these viruses might have the same ancestor although they infect cells from different domains of life (Bamford et al., 2002; Krupovic and Bamford, 2008). Especially the study of thermophiles is important, because their structures stay rather preserved over time. In extreme temperatures mutations are generally highly deleterious. Specific adaptations are required from the thermophilic microbes and losing an important quality for instance, protein thermostability generally leads to the death of the organism (Drake, 2009). Thus, ancient virus types may be found in extremely hot environments (Rissanen et al., 2013).

5.1 P23-77 is a stabile virus

P23-77 is known to be quite stable and it can be stored at room temperature although its optimal temperature for infection is 70 °C (Jaatinen et al., 2008). The stability of P23-77 has been suggested to result from its thermostable capsid proteins. Accordingly, VP16 and VP17 both have melting points over 80 °C (Rissanen et al., 2013). Considering the surroundings, in which P23-77 resides, the protein thermostability is a necessity for survival. In hot springs the temperature may vary and thus, the phage seems to be adapted to high fluctuations in the temperature (Jaatinen et al., 2008). The negative staining

experiments visualized with TEM supported the previous findings. Accordingly, there was little change in the morphology of P23-77 after two weeks of storage at room temperature after the virus purification process. In addition, considering the titers there was no great drop in the infectivity of P23-77 after two weeks of storage either in TV or PBS buffer (data not shown). Although, P23-77 has a superior stability compared to other similar thermophilic phages (Jaatinen et al., 2008) there was a high decrease in the infectivity of P23-77 during rate zonal rate centrifugation (1x-purification) and equilibrium and differential centrifugations (2x-purification) (Table 1). The loss of absolute infectivity is a commonly noticed phenomenon during virus purification (Bourdin et al., 2014). Some viruses might be sensitive to the different purification treatments and lose some of their ability for infection. Particular chemicals utilized in the process might have caused the rupture of virus and the loss of infectivity. In addition, P23-77 has a tendency to aggregate during purification (Jaatinen et al., 2008), which might decrease the infectivity. Yet, the differentially purified virus had high enough specific infectivity value ($1{,}32 \times 10^{12}$ pfu/mg) to be applied in the following experiments (Table 1).

5.1.1 P23-77 morphology changed during the TEM immunolabelling protocol

As stated above, there is commonly a decrease of infectivity and sample material during virus purification. Sometimes it has to be chosen whether high purity or good condition of virus is more relative for the study in question. Difficulties may arise when both of these qualities are required. There was a great difference between the morphology of the immunolabelled 1x- and 2x-purified P23-77 virions visualized with TEM. High purity of the sample was required for TEM, because even minute impurities show under the electron microscope. In addition, when the structure of a bacteriophage is examined, the morphology of the virions should be preserved. The 2x-purified immunolabelled P23-77 virions had lost their spherical appearance and did not contain any DNA. As the negative staining experiments showed, the virus was not ruptured during the 2x-purification. Virions were seen clearly as spherical particles containing DNA (seen as white spots) and the virion morphology was comparable with the results received in the study Jaatinen et al, (2008) (Figure 2a). The change in morphology occurred during the immunolabelling protocol. It would seem that some chemicals utilized in the 2x-purification and the immunolabelling protocol were cross-reacting and affecting the virion morphology. It is possible that cesium chloride was interacting with glutaraldehyde and causing the rupture

of structure. In addition, the aggregation of 2x-purified viruses was noticed, which might arise from the drying of virus during the immunolabelling. In the future, the 2x-purified phages could be dialyzed to remove cesium chloride from the sample. Then, it could be studied if the virions remained intact during the immunolabelling.

5.2 Western blot method for VP11 detection successfully optimized

One aim of the study was to optimize a western blot method for the detection of VP11. The SNAP i.d. 2.0 Protein Detection system was utilized in the immunolabelling optimization (EMD Millipore). The instructions state that high concentration of antibody should be used in combination with short (10 min) incubation times (EMD Millipore). However, these recommended conditions did not give specific signal for VP11. There was a lot of unspecific binding of the antibody, which was seen as dark spots on the background. Thus, it was concluded that the concentration of the anti-VP11 serum should be lower to improve the signal. If too high concentration of antibody is used, the antibody starts to bind unspecifically (Hyatt and Eaton, 1993). After varying the incubation times and concentrations of the antibodies, the optimal conditions were found (Table 2). The optimal western blot and immunodetection conditions were successfully established for mini and large gels.

The transfer of protein was not complete from the SDS-PAGE to the PVDF membrane. In order to enhance the transfer of protein, wet tank blotting was tested but there was no improvement. Due to the time limitations considering the lab experiments, there was no time to optimize the protein transfer step further. In addition, many trials had already been done with the protein transfer in previous studies, so it is probable that the conditions for the transfer cannot be improved. Moreover, there was an adequate amount of protein transferred to the PVDF membrane for the immunolabelling process. In general, the separation of protein is better on big gels than on mini gels (Hames and Rickwood, 1990). Hence, VP11 was run on bigger gels to get a clear and specific signal in western blot. In the future, different kinds of nitrocellulose membranes could be tested to see, if the transfer of protein could be enhanced.

5.2.1 The sensitivity of the anti-VP11 serum

The proper functionality of the anti-VP11 serum had to be verified in order to receive reliable results. False results may occur if the primary antibody is not working correctly.

Primary antibodies produced in two different rabbits (193, 194) were tested in the immunolabelling protocol. Better signal was received with the antibody produced in the rabbit 194. In theory both antibodies (193 and 194) should work with the same efficiency. Yet, there might have been something different in the individual animals used, which caused the difference in the anti-VP11 serum sensitivity. In addition, the antibody 193 might have lost its efficiency during shipment or storage by a chance. During the sensitivity tests, it was noticed that the anti-VP11 serum was not stable after two months storage in 4 °C. One should take this into account when repeating the western blot experiments. Large gels and TEM require generally higher effectiveness of the antibody. Thus, the more sensitive antibody (194) had to be chosen for further studies. The detection limit of the anti-VP11 serum was 50 ng, which is quite low sensitivity for an antibody. For instance, the detection limit of HRP is 1 pg, hence over thousand-fold less protein is needed for the detection (PerkinElmer). The detection sensitivity of the anti-VP11 serum suffered from the poor transfer of protein from the SDS-PAGE to PVDF membrane (Figure 3a). It can be reasoned that the anti-VP11 serum could detect VP11 even in higher dilutions, if the transfer of protein from SDS-PAGE to the PVDF membrane was enhanced.

The detection limit for anti-VP11 serum was tested in the TEM studies. Yet, there was no difference in the amount of protein A-gold labels between the pure VP11 samples treated with different concentration of the primary antibody. The similarity of the results might arise from the fact that the dilution series used for the estimation had too narrow range. If there is enough antibody for each protein on the sample, no difference will arise between the different dilutions. In the future studies higher antibody dilutions (> 1:1000) should be used in order to set the detection limit for anti-VP11 serum for TEM immunolabelling.

5.3 VP11 exists as a dimer in the P23-77 virion

Previous studies of the P23-77 capsid structure had shown that there exists an unidentified penton protein at the virion vertices attaching to the protruding spikes. VP11 was hypothesized to be this unidentified penton protein due to its size (Jaatinen et al., 2008). Yet, the results received in this thesis exclude VP11 as the penton protein. The copy number of VP11 in the capsid was estimated to be 147. For a penton protein occupying the 12 vertices of an icosahedral capsid the copy number should be 60. The received copy

number 147 highly exceeds 60 and implies that VP11 is not a penton protein. Yet, it has to be taken into consideration that the method, which was used to the copy number estimation is not extremely specific. The estimation method is based on the idea that the applied gel stain Coomassie blue attaches to all proteins with the same affinity. In reality this might not be the case. In addition, some error may arise when adjusting the background variables by hand. Although the method is not error free, it gives an approximate estimation. The number 147 is much higher than 60. Hence, it is not probable that the estimation would be twice more than it is in reality. Moreover, the copy numbers of VP16 and VP17 were close to their theoretical values (Figure 4) (Rissanen et al., 2013).

Moreover, the results received from the western blot and immunodetection experiments supported the theory of a VP11 dimer. When 2x-purified virus sample was treated with high concentration (5 %) of β-mercaptoethanol there was only one VP11 band visible on the immunoblotted PVDF membrane. If lower concentration (1 %) was used, there were two protein bands visible (Figure 3). Consequently, the lower concentration (1 %) of β-mercaptoethanol was not enough to reduce the disulfide bridge between two VP11. Thus, VP11 dimers and monomers were showing as two separate bands on the blot (Figure 3b and c). In addition, the highest protein band detected was approximately 50 kD in size, which indicates that no higher multimers were present. Yet, it is possible that higher multimers were below the detection limit of the method and thus, were not detected. The size of VP11 monomer is estimated to be 22 kD (Jalasvuori et al., 2009). In the SDS-PAGE VP11 was in line with 25 kD molecular marker band (Figure 3). For a dimer the size would be 50 kD, which would fit rather well with the results. If VP11 would exist as a penton protein, there should have been larger proteins than 50 kD on the blot.

5.4 VP11 location inside the virus capsid

According to the TEM immunolabeling results, it seems that VP11 localizes between the internal lipid membrane and the capsid shell. There was no reaction noticed between the immunolabelled P23-77 virions and protein A gold particles in the TEM experiments (Figure 2b). The immunolabelling of particles enables the examination of a specific protein in a virus sample (Hyatt and Eaton, 1993). Protein A gold was used as a secondary antibody to recognize anti-VP11 serum (194), which attaches to VP11. Consequently, the protein A gold particles served as markers for VP11. Due to the lack of attachment of

protein A gold particles on the virions, it can be assumed that there was no VP11 on the outer capsid shell. The specificity experiments done with anti-VP11 serum showed that the antibody was recognizing VP11 specifically (see 4.2.2). Yet, it has to be considered that the anti-VP11 serum might not recognize VP11 in the mature virus particles although the proteins would reside outside the capsid. Steric hindrance may affect the function of antibodies (Voorhout et al., 1986). If there is some structural component on the virion capsid, which blocks the binding sites of VP11, no reaction occurs. Thus, there would be no markers seen on the virion. In the future, the virions could be treated with low pH or chemicals to loosen the capsid structure. Hypothetically, if VP11 was located below the protein capsid, it would be released to the surroundings after the treatment. In the previous studies the capsid proteins have been liberated from the capsid by a treatment of pH 6.0 or denaturing agents (Jalasvuori et al., 2009). Thus, it could be studied whether there is an increase of antibody labels near the virions after the capsid structure is demolished. In the following studies, it would be important to optimize the immunolabelling protocol for P23-77 for TEM visualization.

5.5 VP11 potential in biochemical applications

Many extremophilic proteins have been applied in industrial and biochemical applications (Air and Harris, 1974; Song and Zhang, 2008). Especially the thermostability of proteins is a highly appreciated quality, which is needed in many industrial and biochemical applications (Frock and Kelly, 2012). Proteins acquired from thermophiles generally work faster and are more resistant to environmental stress than proteins isolated from mesophilic (25-50 °C) organisms (for review see Vieille and Zeikus, 2001). For instance, in the study performed by Song and Zhang (2008) a new thermostable non-specific nuclease was found from a thermophilic bacteriophage GBSV1. The study suggests that the nuclease could be used in the determination of nucleic acid structure, the removal of nucleic acids during protein purification and the use as an antiviral agent (Song and Zhang, 2008).

Although VP11 does not possess enzymatic activity, it potentially has another qualities, which could be utilized in biochemical applications. As the MCPs, VP11 has been has been shown to be rather thermostable and withstand partial unfolding (Rissanen I., 2014; Pawlowski et al., 2015). In addition, the preliminary studies performed by Pawlowski et al. (2015) suggest that VP11 might possess membrane binding potential. VP11 could trigger

the capsid assembly by interacting with the lipid membrane, which results in the binding of MCPs (Pawlowski et al., 2015). The location of VP11 between the internal lipid membrane and the capsid shell would be in line with this suggestion. Moreover, VP11 has been shown to bind nucleic acids without sequence specificity (Pawlowski et al., 2015). These qualities could be highly useful in many biochemical applications requiring either thermostability or association with nucleic acids or membranes. Yet, a great number of studies have to be performed before VP11 may be used in any application. In the future, more studies are needed to determine the specific function of VP11 and to gain information about its intriguing qualities.

6. Summary

P23-77 is a quite recently found bacteriophage (Yu et al., 2006) and its detailed capsid structure is unknown. The results received during this thesis gave valuable information about the location of the minor capsid protein VP11. In addition, a western blot method for VP11 detection was optimized successfully and it can be used in the following studies. It was confirmed that the anti-VP11 serum produced in two different rabbits (193 and 194) was working correctly and can be used in future studies. The anti-VP11 serum produced in the rabbit 194 was noticed to be more sensitive than 193. Thus, the anti-VP11 serum 194 was chosen for following studies. Highly infectious P23-77 virus particles ($1{,}32 \times 10^{12}$ pfu/mg) were successfully propagated and purified for the following TEM and western blot studies. In line with the previous studies performed, the bacteriophage P23-77 was noticed to be rather stable. There was no change in the morphology of the virus after two weeks of storage at room temperature in TV-buffer. The copy number of VP11 was estimated to be 147, which excludes the possibility of a penton protein. Supporting the results, the western blot studies showed only monomer and dimer structures for VP11, which indicate that VP11 exist as a dimer. Moreover, the preliminary TEM immunolabelling studies showed that VP11 was not recognized outside the virion capsid. Accordingly, the results point to the direction that VP11 is a dimer protein inside the virion capsid. VP11 is estimated to function as a linker between the internal lipid membrane and the capsid shell due to its suggested location and other qualities. VP11 might have potential in biochemical applications, but more studies are needed to reveal its function.

7. References

Abrescia, N. G. A., J. J. B. Cockburn, J. M. Grimes, G. C. Sutton, J. M. Dispose, S. J. Butcher, S. D. Fuller, C. San Martin, R. B. Burnett, D. I. Stuart, D. H. Bamford, and J. K. H. Bamford. 2004. Insights into assembly from structural analysis of bacteriophage PRD1. *Nature.* 432:68-74.

Abrescia, N. G., J.M. Grimes, H. M. Kivelä, R. Assenberg, G. C. Sutton, S. J. Butcher, J. K. Bamford, D. H. Bamford, and D. I. Stuart. 2008. Insights into virus evolution and membrane biogenesis from the structure of the marine lipid-containing bacteriophage PM2. *Mol. Cell.* 31:749-761.

Air, G. M., and J. I. Harris. 1974. DNA-dependent RNA polymerase from the thermophilic bacterium *Thermus aquaticus*. *FEBS Lett.* 38:277-281.

Amersham Biosciences, EMD Millipore. 2012. Protein Blotting Handbook, 6th ed, EMD Millipore, Billerica, MA

Bamford, J. K., and D. H. Bamford. 1990. Capsomer proteins of bacteriophage PRD1, a bacterial virus with a membrane. *Virology*. 177 (2):445-51

Bamford, D. H, R.M Burnet and D. I. Stuart. 2002. Evolution of viral structure. *Therm Popul Biol*. 61(4): 461-70.

Benson, S. D, J. K Bamford, D. H. Bamford, and R. M. Burnett. 1999. Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. *Cell.* 98(6):825-33.

Bourdin, G., B. Schmitt, G. L. Marvin, J. E. GErmond, S. Zuber, L. Michot, G. Reuteler, and H. Brussow. 2014. Amplification and purification of T4-like *Escherichia coli* phages for phage therapy: from laboratory to pilot scale. *Appl. Environ. Microbiol.* 80:1469-1476.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Breitbart, M., L. Wegley, S. Leeds, T. Schoenfeld, and F. Rohwer. 2004. Phage community dynamics in hot springs. *Appl. Environ. Microbiol.* 70:1633-1640.

Bull, J. J., M. R. Badgett, and H. A. Wichman. 2000. Big-Benefit Mutations in a Bacteriophage Inhibited with Heat. *Molecular Biology and Evolution.* 7:17(6):942-50.

Canganella, F., and J. Wiegel. 2011. Extremophiles: from abyssal to terrestrial ecosystems and possibly beyond. *Naturwissenschaften.* 98:253-279.

Caspar D. L., and A. Klug. 1962. Physical principles in the construction of regular viruses. *Cold Spring Harb Symp Quant Biol*. 27:1-24

Cox, J., A. M. Schubert, M. Travisano, and C. Putonti. 2010. Adaptive evolution and inherent tolerance to extreme thermal environments. *BMC Evol. Biol.* 10:75-2148-10-75.

Drake, J. W. 2009. Avoiding Dangerous Missense: Thermophiles Display Especially Low Mutation Rates. *Plos Genet.* 5(6) e1000520.

Frock, A. D., and R. M. Kelly. 2012. Extreme Thermophiles: Moving beyond single-enzyme biocatalysis. *Curr. Opin. Chem. Eng.* 1:363-372.

Gowen, B., J. K. Bamford, D.H. Bamford, and S.D. Fuller. 2003. The tailless icosahedral membrane virus PRD1 localizes the proteins involved in genome packaging and injection at a unique vertex. *J. Virol.* 77:7863-7871.

Grigorian, A. L., J. J. Bustamante, P. Hernandez, A.O. Martinez, and L.S. Haro. Extraordinarily stable disulfide-linked homodimer of human growth hormone. 2005. *Protein Sci.* 14(4):902-13

Hames, B. D., and D. E. Rickwood. 1990. Gel Electrophoresis of Proteins: a Practical Approach. Essential cell biology, Volume 1. Cell structure. J. Davey and M. Lord, editors. Oxford University Press, New York. 197-268

Hassan J., C. Feighery, and A. Whelan. 1987. Staining of molecular weight markers on nitrocellulose using Ponseau S. *J. Clin. Lab. Immunol.* 24 (2):104

Horikoshi, K., G. Antranikian, A. T. Bull, F. T. Robb, and K. O., Stetter. 2010. Extremophiles handbook. Springer Science & Business Media, New York. 4-12.

Hyatt, A. D. and, B. Eaton. 1993. Immuno-Gold Electron Microscopy in Virus Diagnosis and Research. CRC press, Florida. 79pp

Jaatinen, S. T., L. J. Happonen, P. Laurinmaki, S. J. Butcher, and D. H. Bamford. 2008. Biochemical and structural characterisation of membrane-containing icosahedral dsDNA bacteriophages infecting thermophilic *Thermus thermophilus*. *Virology*. 379:10-19.

Jalasvuori, M., and J. K. Bamford. 2008. Structural co-evolution of viruses and cells in the primordial world. *Orig. Life Evol. Biosph*. 38:165-181

Jalasvuori, M., S. T. Jaatinen, S. Laurinavicius, E. Ahola-Iivarinen, N. Kalkkinen, D. H. Bamford, and J. K. Bamford. 2009. The closest relatives of icosahedral viruses of thermophilic bacteria are among viruses and plasmids of the halophilic archaea. *J. Virol.* 83:9388-9397.

Jäälinoja, H.T., E. Roine, P. Laurinmaki, H. M. Kivela, D. H. Bamford, and S. J. Butcher. 2008. Structure and host-cell interaction of SH1, a membrane-containing, halophilic euryarchaeal virus. *Proc. Natl. Acad. Sci. U.S.A.* 105:8008-8013.

Keen, E. C. 2015. A century of phage research: bacteriophages and the shaping of modern biology. *Bioessays.* 37:6-9.

Kim, J. J., H. N. Kim, R. Masui, S. Kuramitsu, J. H. Seo, K. Kim, and M. H. Sung. 2008. Isolation of uncultivable anaerobic thermophiles of the family *Clostridiaceae* requiring growth-supporting factors. *J.Microbiol.Biotechnol.* 18:611-615.

Krupovic, M., and D. H. Bamford. 2008. Archaeal proviruses TKV4 and MVV extend the PRD1-adenovirus lineage to the phylum *Euryarchaeota*. *Virology*. 375(1):292-300.

Maaty, W. S., A. C. Ortmann, M. Dlakic, K. Schulstad, J. K. Hilmer, L. Liepold, B. Weidenheft, R. Khavat, T. Douglas, M. J. Young, and B. Bothner. 2006. Characterization of the archaeal thermophile *Sulfolobus* turreted icosahedral virus validates an evolutionary link among double-stranded DNA viruses from all domains of life. *J Virol.* 80(15):7625-35.

Matsushita, I., H. Yanase. 2009. The genomic structure of thermus bacteriophage {phi}IN93. *J Biochem.*146(6):775-85.

Monera, O., D., Kay, C., M., and Hodges R.S. 1994. Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. *Protein Science*, 3:1984-1991.

Olkkonen, V. M. and, D. H. Bamford. 1989. Quantitation of the adsorption and penetration stages of bacteriophage phi 6 infection. *Virology*. 171(1):229-38

Pawlowski, A., A. M. Moilanen, I. A. Rissanen, J. A. Maatta, V. P. Hytonen, J. A. Ihalainen, and J. K. Bamford. 2015. The minor capsid protein VP11 of thermophilic bacteriophage P23-77 facilitates virus assembly using lipid-protein interactions. *J. Virol.* 15: 7593-7603

Pawlowski, A., I. Rissanen, J. K. Bamford, M. Krupovic, and M. Jalasvuori. 2014. Gammasphaerolipovirus, a newly proposed bacteriophage genus, unifies viruses of halophilic archaea and thermophilic bacteria within the novel family *Sphaerolipoviridae*. *Arch. Virol.* 159:1541-1554

Pietilä, M. K., P. Laurinmaki, D. A. Russell, C. C. Ko, D. Jacobs-Sera, S. J. Butcher, D. H. Bamford, and R. W. Hendrix. 2013. Insights into head-tailed viruses infecting extremely halophilic archaea. *J. Virol.* 87:3248-3260.

Porter, K., P. Kukkaro, J. K. Bamford, C. Bath, H. M. Kivelä, M. L Dyall-Smith, D. H. Bamford. 2005. SH1: A novel, spherical halovirus isolated from an Australian hypersaline lake. *Virology*. 335(1):22-23.

Rissanen, I. 2014. Ph.D dissertation. An ancient virus type from extreme environments. University of Jyväskylä, Jyväskylä.

Rissanen, I., J. M. Grimes, A. Pawlowski, S. Mantynen, K. Harlos, J. K. Bamford, and D. I. Stuart. 2013. Bacteriophage P23-77 capsid protein structures reveal the archetype of an ancient branch from a major virus lineage. *Structure.* 21:718-726.

Schagger, H., and G. Von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem.* 1:166(2):368-79.

Song, Q., and X. Zhang. 2008. Characterization of a novel non-specific nuclease from thermophilic bacteriophage GBSV1. *BMC Biotechnology.* 8:43

Strömsten N. J, D. H. Bamford, and J. K. Bamford. 2005. In vitro DNA packaging of PRD1: a common mechanism for internal-membrane viruses. *J Mol Biol.* 348(3):617-29.

Vieille, C. and G.J. Zeikus. 2001. Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. *Microbiol. Mol. Rev.* 65:1-43.

Voorhout, W.F., J. J. Leunissen-Bijvelt, J .L. Leunissen, and A. J. Verkleij. 1986. Steric hindrance in immunolabelling. *J. Microsc.* 141:303-310.

Wall, K., J. Cornell, R. W. Bizzoco, and S. T. Kelley. 2015. Biodiversity hot spot on a hot spot: novel extremophile diversity in Hawaiian fumaroles. *MicrobiologyOpen*. 4 (2):267-281.

Yu, M. X., M. R. Slater, and H. Ackermann. 2006. Isolation and characterisation of *Thermus* bacteriophages. *Arch Virol*. 151(4): 663-679.

Attachments

Appendix 1: *Thermus* media and buffer

T. thermophilus liquid medium (TM)

4 g yeast extract
8 g polypeptone
2 g NaCl

Ingredients were diluted in 1 L of destilled water by magnetic stirring. Sodium hydroxide (NaOH) was used to adjust the pH to 7.5. Media was sterilized by autoclaving.

TM soft-agar and –agar plates

4 g yeast extract
8 g peptone
2 g NaCl

Ingredients were diluted in 1 L of destilled water by magnetic stirring. NaOH was used to adjust the pH to 7.5. For 1 L of soft-agar, 7 g of agar powder was weighted. For TM plates the needed amount was 20 g of agar powder. All liquids were autoclaved.

TV-buffer

20 mM Tris-HCl (pH 7.5)
150 mM NaCl
5 mM $MgCl_2$

Ingredients were diluted in sterile water and mixed well. TV-buffer was always freshly prepared from stock solutions of the ingredients listed above.