Entry of the membrane-containing bacteriophages into their hosts

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biosciences, University of Helsinki, in the auditorium 3 of Info center Korona, Viikinkaari 11, Helsinki, on June 18th, at 8 a.m.

HELSINKI 2010

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ISBN 978-952-10-6280-3 (paperback)
ISBN 978-952-10-6281-0 (PDF)
ISSN 1795-7079

Yliopistopaino, Helsinki University Printing House
Helsinki 2010

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. Daugelavičius R, Cvirkaitė V, Gaidelytė A, Bakienė E, Gabrėnaitė-Verkhovskaya R, Bamford DH. 2005. Penetration of enveloped double-stranded RNA bacteriophages phi13 and phi6 into *Pseudomonas syringae* cells. *J Virol.* 79(8):5017-26.

II. Gaidelytė A*, Cvirkaitė-Krupovič V*, Daugelavičius R, Bamford JK, Bamford DH. 2006. The entry mechanism of membrane-containing phage Bam35 infecting *Bacillus thuringiensis*. *J Bacteriol.* 188(16):5925-34.

III. Cvirkaitė-Krupovič V, Krupovič M, Daugelavičius R, Bamford DH. 2010. Calcium ion-dependent entry of the membrane-containing bacteriophage PM2 into *Pseudoalteromonas* host. *Virology*. In press.

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SUMMARY

Viruses are biological entities able to replicate only within their host cells. Accordingly, entry into the host is a crucial step of the virus life-cycle. The focus of this study was the entry of bacterial membrane-containing viruses into their host cells. In order to reach the site of replication, the cytoplasm of the host, bacterial viruses have to traverse the host cell envelope, which consists of several distinct layers. Lipid membrane is a common feature among animal viruses but not so frequently observed in bacteriophages. There are three families of icosahedral bacteriophages that contain lipid membranes. These viruses belong to families *Cystoviridae, Tectiviridae*, and *Corticoviridae*. During the course of this study the entry mechanisms of phages representing the three viral families were investigated. We employed a range of microbiological, biochemical, molecular biology and microscopy techniques that allowed us to dissect phage entry into discrete steps: receptor binding, penetration through the outer membrane, crossing the peptidoglycan layer and interaction with the cytoplasmic membrane. We determined that bacteriophages belonging to the *Cystoviridae*, *Tectiviridae*, and *Corticoviridae* viral families use completely different strategies to penetrate into their host cells.

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A. LITERATURE REVIEW

1. Introduction

Viruses are biological entities able to replicate only within their host cells. They are the most abundant group of organisms on Earth (in total 1031-1032 virus particles in the biosphere), outnumbering their hosts at least by a factor of ten (Bergh et al., 1989; Hendrix, 2002; Wommack and Colwell, 2000). Viruses infect organisms from all domains of life (Eukarya, Archaea and Bacteria). The virion, the extracellular form of a virus, is composed of at least two components – the viral genome and the protective shell, called a capsid. The genome of a virus can be either RNA or DNA, double-stranded or single-stranded, circular or linear, with or without a terminal protein (Ackerman, 2006). Viral genome contains all the information needed for its replication in the host cell and for directing synthesis of structural components of the virion. The capsid of a virion serves as a vehicle to move from one cell to another. The capsid possesses a dual character, stability and lability. In the harsh extracellular environment it has to be stable and protect the genome. On the other hand, after binding to the susceptible host the capsid has to disassemble in order to ensure the success of genome delivery into the replication-competent intracellular environment. Viruses infecting bacterial cells are called bacteriophages (eating bacteria) or simply phages. Bacteriophages were discovered in the beginning of the twentieth century independently by two scientists, Frederick Twort and Felix d'Herelle. The bacteriophages are widely spread and have been isolated from various environments such as oceans (Espejo and Canelo, 1968b), Arctic sea ice (Borriss et al., 2003), hot springs (Breitbart et al., 2004; Yu et al., 2006), sewage (Olsen et al., 1974), agricultural and forest soil samples (Williamson et al., 2005), or the Sahara desert (Prigent et al., 2005). Based on the type of nucleic acid and virion morphology bacteriophages are classified by ICTV (International Committee on Taxonomy of Viruses) into ten families (Fauquet et al., 2005). The characteristics of bacterial virus families are summarized in Table 1. The majority of the described phages (96%) have an icosahedral capsid and a tail, while the rest are tailless icosahedral, filamentous or pleomorphic (Fig. 1).

Table 1. Overview of bacterial virus families.

Family	Capsid morphology	Additional features	Genome type	Examples
Myoviridae	icosahedral	tail (contractile)	dsDNA, L	T4
Siphoviridae	icosahedral	tail (long non-contractile)	dsDNA, L	λ
Podoviridae	icosahedral	tail (short non-contractile)	dsDNA, L	T7
Tectiviridae	icosahedral	internal membrane	dsDNA, L	PRD1
Corticoviridae	icosahedral	internal membrane	dsDNA, C	PM2
Plasmaviridae	pleomorphic	enveloped	dsDNA, C	L2
Microviridae	icosahedral	nonenveloped	ssDNA, C	ϕX174
Inoviridae	filamentous	long flexible or short rigid	ssDNA, C	M13
Leviviridae	icosahedral	nonenveloped	ssRNA, L	MS2
Cystoviridae	icosahedral	enveloped, multilayered	dsRNA, L, S	ϕ6

L, linear; C, circular; S, segmented.

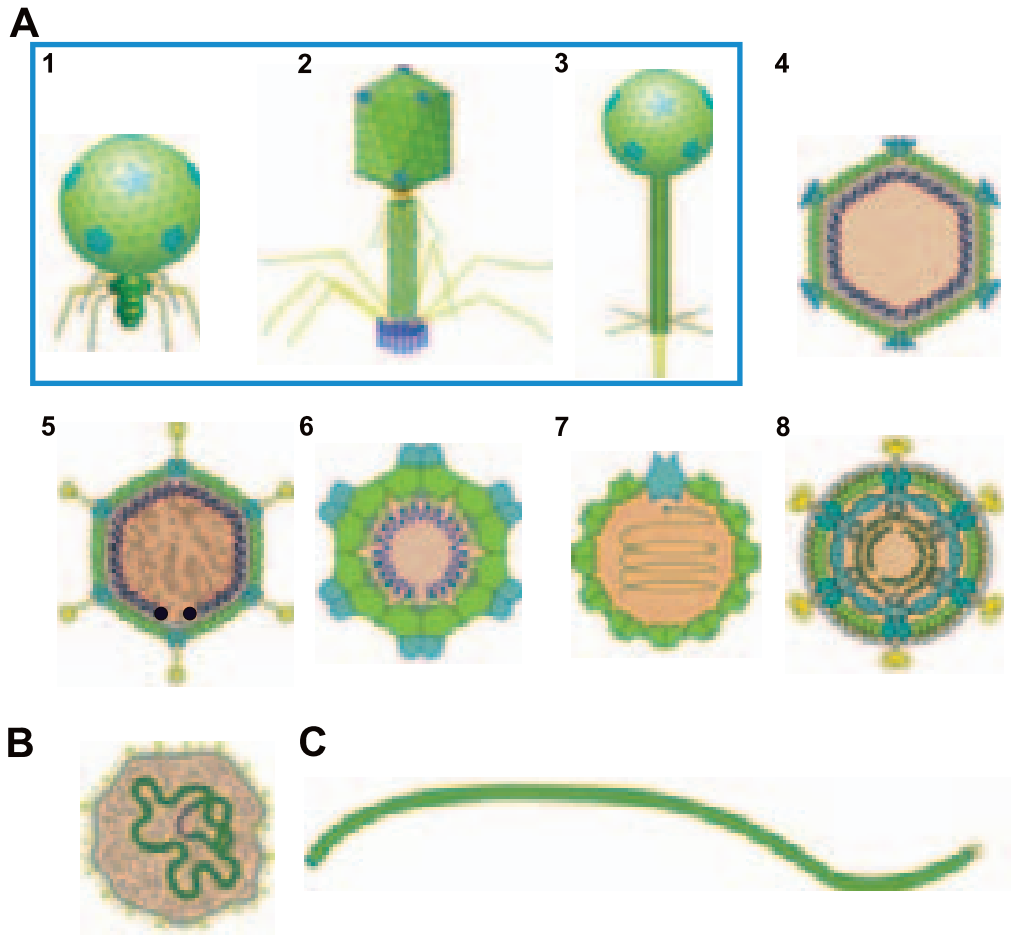


Figure 1. Examples of different virion morphotypes. (A) Icosahedral virions. Tailed bacteriophages of *Podoviridae* (1); *Myoviridae* (2); *Siphoviridae* (3) families are boxed in blue. Internal membrane containing phages of *Corticovidae* (4) and *Tetctiviridae* (5) families; 6, *Leviviridae*; 7, *Microviridae*; Enveloped icosahedral phages of the *Cystoviridae* (8) family. (B) Pleomorphic virion of bacteriophages of the family *Plasmaviridae*. (C) Flexible filamentous capsid of bacteriophages belonging to the *Inoviridae* family. Viruses are not drawn to scale. All pictures were adapted with permission from ViralZone, Swiss Institute of Bioinformatics (www.expasy.ch/viralzone).

Being quite simple and easy to culture, bacteriophages are excellent model systems to study such biological processes as the assembly of macromolecular structures, molecular mechanisms for genome replication and packaging. Phages are also used in practical applications, such phage therapy, and phage display (Clark and March, 2006). The focus of this thesis is the entry of the membrane-containing bacteriophages into their bacterial hosts.

2. Bacteriophage entry

2.1 Bacterial cell envelope – the barrier for phage entry

2.1.1 Gram-negative bacteria

The complex and multilayer cell envelope of gram-negative bacteria consists of two membranes – the outer (OM) and the cytoplasmic membrane (CM) – and a thin peptidoglycan layer sandwiched between them (Fig. 2A).

The OM is an asymmetric bilayer – the inner leaflet is composed of phospholipids, while the outer one mainly consists of lipopolysaccharides (LPS). LPS comprises 20-40% of the OM weight (Nikaido, 1999). The LPS can be structurally divided into three parts: a lipid moiety known as the lipid A, a non-repeating "core" oligosaccharide, and the outermost part – a hydrophilic polysaccharide called the O-antigen which differs from species to species (Fig. 2B; Osborn et al., 1964). Approximately 2% of the proteins that are encoded by the bacterial genome are found in the outer membrane. The hallmark feature of the OM transmembrane proteins is that they possess multiple β-strands and form barrel-like structures (Schulz, 2002; Xie and Dalbey, 2008).

The primary function of the OM is to act as a permeability barrier for cytotoxic compounds and to control the access of solutes to the periplasm and CM. To ensure the transport of necessary compounds the OM contains a number of transmembrane channels, transporters. These can be either nonspecific, allowing passive diffusion of low-molecular mass solutes, or specific, ensuring permeation of oligosaccharides or certain solutes (exemplified by vitamin B_{12} and iron siderophores) that are large in size and likely to be present in the medium at low concentrations (Osborn and Wu, 1980).

The space between the OM and the CM is called periplasm. The peptidoglycan (also known as murein layer, murein sacculus or cell wall) resides in the periplasmic space and is covalently linked to the OM by Braun's lipoproteins (Braun and Sieglin, 1970; Costerton et al., 1974). The peptidoglycan is an essential component of the bacterial cell wall and is the largest single cellular molecule. It consists of linear glycan chains composed of two different alternating units: *N*-acetyl-glucosamine (NAG) and *N*-aceyl-muramic acid (NAM) linked via the β-1,4-glycosidic bond (Fig. 2C). These glycan chains are cross-linked together by short (three to five amino acids) peptides attached to the N-aceylmuramic acid thus forming a rigid three-dimensional network (Green, 2002). The peptidoglycan has an effective pore of 5 nm in diameter and permits the diffusion of globular proteins of up to 50 kDa in size (Demchick and Koch, 1996). In addition to the molecular sieve function, the peptidoglycan layer plays an important role in i) resisting the internal pressure of the cell, ii) maintaining the specific size and shape of the cell, iii) and serving as an anchor for diverse proteins (Dmitriev et al., 1999). The thickness of the peptidoglycan layer in gram-negative bacteria varies from 2.5 to 7 nm (Yao et al., 1999). The cytoplasmic membrane of gram-negative bacteria is composed of phospholipids (30–60% by weight) that form a bilayer and proteins. For most bacteria the predominant zwitterionic phospholipid is phosphatidylethanolamine (PE), while the predominant anionic lipids of the CM are phosphatidylglycerol (PG) and cardiolipin (CL; Epand and Epand, 2009). 20–30% of the proteins that are encoded by the bacterial proteome are CM proteins. Proteins of the cytoplasmic membrane are either integral, containing one or multiple α-helical transmembrane regions, or are

peripherally associated with it (Xie and Dalbey, 2008). The CM possesses proteins that are involved in respiration, oxidative phosphorylation, phospholipid and peptidoglycan biosynthesis, transport of small and large molecules as well as in transmission of environmental signals across the membrane (Kadner, 1996).

2.1.2 Gram-positive bacteria

Gram-positive and gram-negative bacteria differ considerably in the structure of their cell envelopes (Fig. 2D). First of all, gram-positive cells do not possess an outer membrane. Secondly, their cell wall is considerably thicker – highly crosslinked, three dimensional mesh-like structure. Finally, gram-positive cells have additional cell wall glycopolymers (CWGs) that are either attached to the peptidoglycan or to the cytoplasmic membrane. Peptidoglycan-anchored CWGs are teichoic acids, teichuronic acids, cell wall polysaccharides and arabinogalactans, while lipoteichoic acids, lipomannans, and lipoarabinomannans belong to the membrane-anchored CWGs. It has been proposed that CWGs are important in protecting the gram-positive cell (Weidenmaier and Peschel, 2008). They anchor the S layer proteins and clog the peptidoglycan pores, this way modifying the physicochemical properties of the cell wall to slow down the passage of harmful substances such as bacteriocins, antibiotics, and surfactants (Weidenmaier and Peschel, 2008).

2.2 Adsorption

The first stage in virus infection is recognition of the susceptible host cell. The initial recognition is accomplished by highly specific interactions between the virus and its host. The viral receptor-binding proteins are adapted to specifically bind to structures exposed on the surface of a susceptible cell (Haywood, 1994). Phages use a plethora of cellular structures as their receptors. Some phages have more than one receptor (Monteville et al., 1994). It is also possible that the receptor exists in several conformations: i) binding the phage reversibly, ii) irreversibly or iii) does not bind phage particles at all (Schwartz, 1980).

2.2.1 Cellular receptors

A number of bacteriophages infecting gram-negative bacteria are known to recognize LPS as their receptor. The receptor structure involved in actual phage binding is usually only a small segment within the whole LPS molecule. The R-core of the lipopolysaccharide is the receptor for phages such as P1, T4, TLS and ϕ13 (German and Misra, 2001; Goldberg et al., 1997; Qiao et al., 2000). The O-antigen of LPS serves as a receptor for bacteriophages ϕYeO3-12 and ϕX174 (al-Hendy et al., 1991; Feige and Stirm, 1976; Jazwinski et al., 1975b). The outer core oligosaccharide of *Y. enterocolitica* O:3 serves as a receptor to phage ϕR1-37 (Kiljunen et al., 2005).

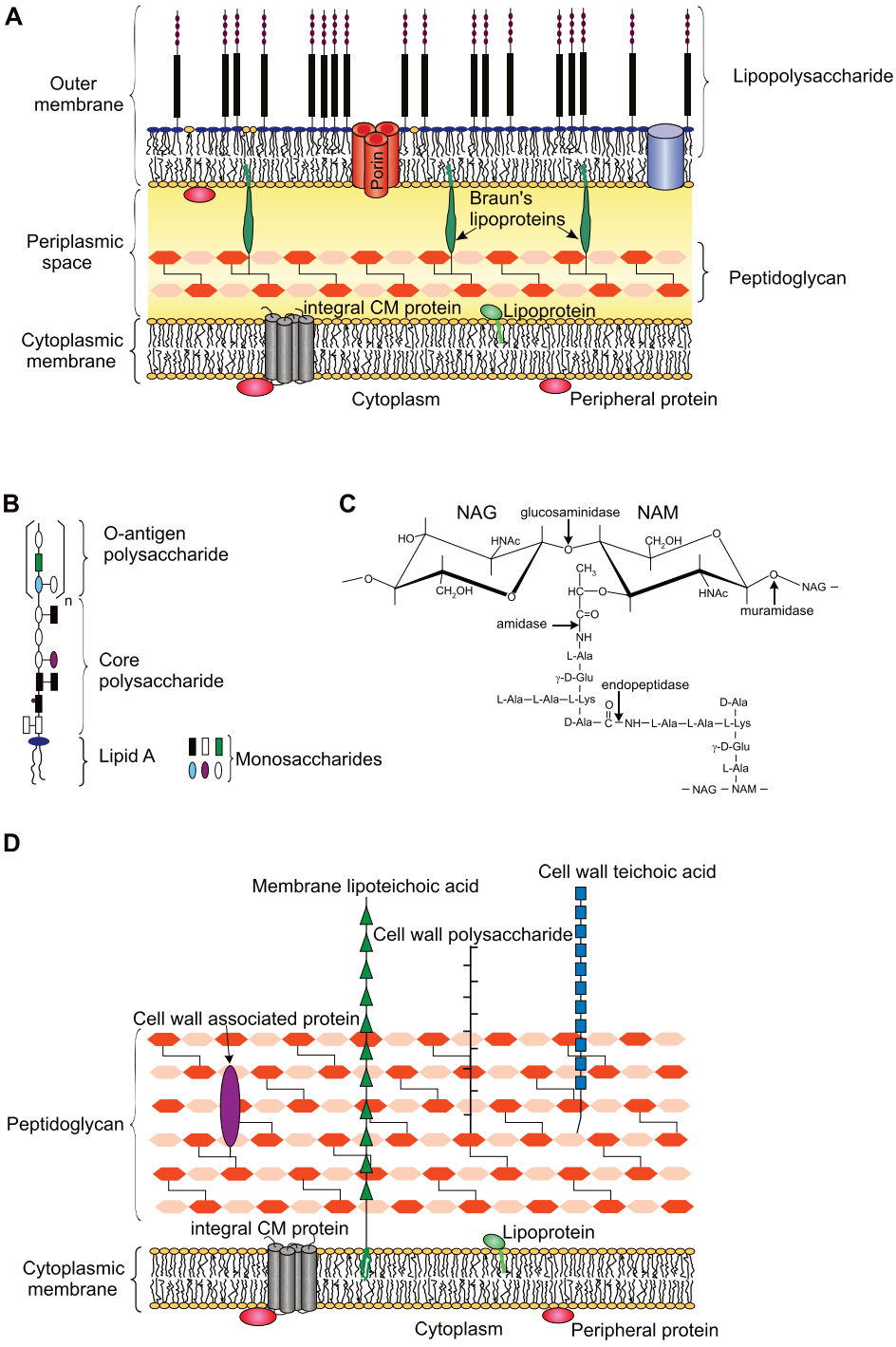


Figure 2. The bacterial cell envelope. A. Schematic representation of gram-negative cell envelope. B. Schematic structure of LPS. C. Peptidoglycan layer. Potential lysin cleavage sites are indicated by arrows. D. Schematic representation of gram-positive cell wall.

The outer membrane protein complexes such as porins or transporters are another type of molecules recognized by phages infecting gram-negative bacteria. For example, tailed bacteriophages T1, ϕ80, and T5 adsorb to FhuA, a protein complex involved in transport of ferric-iron (Braun et al., 2002; Braun and Wolff, 1973; Hancock and Braun, 1976). Other bacteriophages, such as C1 and BF23, use BtuB, the OM vitamin B12 transporter, for adsorption (Bassford et al., 1977; Bradbeer et al., 1976). Bacteriophage H8 uses FepA, the ferric enterobactin receptor, for binding to the host (Rabsch et al., 2007). T4, T2, K3, PP01 all employ outer membrane porins as binding site on the host surface (Henning and Hashemol-Hosseini, 1994; Morita et al., 2002; Wood et al., 1994).

A number of phages also adsorb to long filamentous structures known as pili. Bacteriophages ϕ6 and CTXΦ attach to the type IV pilus, filamentous ssDNA phages fl, fd, and M13 and ssRNA phage Qβ bind to the F-pili. Bacteriophage Ike use N or O pili for binding to the host surface (Bamford et al., 1976; Paranchych, 1975; Russel and Model, 2006). Some bacteria have long, thread-like appendages called flagella, which are responsible for motility of these bacterial cells. Bacteriophages SP3, PBS1, χ use these structures as their receptors (Frankel and Joys, 1966; Schade et al., 1967; Shea and Seaman, 1984).

Bacteriophages infecting gram-positive bacteria can use peptidoglycan, teichoic/lipoteichoic acids or even proteins of the cytoplasmic membrane as their receptors. Phages sk1, jj50, 64, and A511 were shown to bind to the peptidoglycan (Geller et al., 2005; Wendlinger et al., 1996). Bacteriophages ϕ25 and ϕ29 use teichoic acid components as receptors (Young, 1967), while lactobacillus phages LL-H and JCL1032 bind to the lipoteichoic acids constituents (Räisänen et al., 2004).

The lipoprotein fraction isolated from *Streptococcus lactis* had receptor activity for bacteriophage ml3 (Oram, 1971). *Bacillus subtilis*-infecting SPP1 first binds reversibly to teichoic acids, and then for irreversible adsorption uses ectodomain of the CM protein YueB (Baptista et al., 2008; Sao-Jose et al., 2004).

2.2.2 Bacteriophage receptor-binding proteins (anti-receptors)

The binding of phages to the receptors present on the host cell surface is mediated by receptor-binding proteins, or anti-receptors, that are exposed on the surface of the virion. In most cases, anti-receptors are homomultimeric proteins and have a modular structure. Usually the domains that connect anti-receptors with the virions are conserved among the members within a viral family, while the receptor binding domain sequences are more diverged, adapted to recognize a variety of receptors, and determine the host specificity.

Filamentous bacteriophages (fd, M13, f1, Ike, CTXΦ) at one end of the virion contain the minor coat protein pIII. The two N-terminal domains (N1 and N2) of pIII mediate phage binding to its cellular receptor (Stengele et al., 1990). The binding of N2 domain to the pilus leads to a conformational change in pIII resulting in exposure of the N1 domain to the secondary receptor, the TolA protein (Lubkowski et al., 1999; Riechmann and Holliger, 1997).

The spike proteins of membrane-containing bacteriophages (PM2, PRD1, ϕ6) serve as receptor-binding proteins. The N-terminus of pentameric protein P1 of corticovirus PM2 was shown to be responsible for receptor recognition and binding (Abrescia et al., 2008). Bacteriophage PRD1 has two separate spikes formed by proteins P2 and P5 that

protrude from the fivefold vertices of the icosahedral capsid (Huiskonen et al., 2007). However, only P2 has a receptor-binding activity (Mindich et al., 1982). Cystovirus ϕ6 binds to its receptor via spike protein P3 (Bamford et al., 1987; Romantschuk and Bamford, 1985).

Icosahedral tailless phage ϕX174 spike proteins G and H, present at the fivefolds, are responsible for the host recognition and binding (Kawaura et al., 2000; Suzuki et al., 1999). Icosahedral ssRNA bacteriophages, belonging to the *Leviviridae* virus family, bind to the receptor via a maturation protein. Interestingly, leviviruses contain only one copy of the maturation protein per virion (Van Duin and Tsareva, 2006).

Phages belonging to the *Podoviridae* family (phages P22, phi29, P2, epsilon15, T7) bind to the cellular receptors via homotrimeric tail fibers. Three to six tail fibers bound to the short phage tail are required for the infectivity. The receptor-binding site is located in the central part of the tail fiber protein. Interestingly, it has been demonstrated that some tail fibers also have receptor-destroying (endoglycosidase) activity that would facilitate the penetration of the phage through the polysaccharide capsule closer to the bacterial membrane; it is also likely that the latter activity functions to facilitate progeny release from the cell debris after the host cell lysis (Steinbacher et al., 1996). Curiously, another podovirus ϕK1-5 contains two sets of tail fibers. One set is used to adsorb to the type K1, while the other one, to the K5 capsule of the host (Scholl et al., 2001).

The anti-receptor proteins of phages belonging to *Siphoviridae* and *Myoviridae* families are located at the tips of their long tails that are distal to the capsid. The receptor-binding proteins of siphoviruses p2 and TP901-1 are homotrimers (Spinelli et al., 2006a; Spinelli et al., 2006b). The receptor binding activity of SPP1 is associated with the tip of the tail, however, the identity of the viral protein responsible for receptor–anti-receptor interaction remains unknown (Plisson et al., 2007).

The binding of the myovirus T4 to the host is mediated by long and short tail fibers. The adsorption of at least three long tail fibers to the receptors leads to the interaction of the homotrimeric short fibers (protein gp12) with the cellular receptor (Kanamaru et al., 2002; Riede, 1987; Thomassen et al., 2003).

2.3 Genome penetration through the cell envelope

Interaction between the phage receptor-binding protein and the receptor structure on the bacterial cell surface leads to irreversible conformational changes in the receptor-binding protein followed by further rearrangements in the virion structure. The rigid structure of the phage capsid is destabilized to facilitate genome penetration. The majority of studied bacteriophages, when entering into the host, deliver only their genomes along with necessary factors leaving the rest of the capsid outside, associated with the cell surface (for review see Poranen et al., 2002). Bacteriophages utilize different mechanisms to enter the host. The entry strategies can be divided into three main types, (i) genome delivery through a vertex, (ii) coat dissociation at the cell envelope, and (iii) fusion and endocytosis-like pathway.

2.3.1 Peptidoglycan digestion

For the successful entry into the host cell, an incoming bacteriophage has to overcome a barrier formed by the peptidoglycan layer. For this purpose phages employ muralytic enzymes that create transient openings in the murein sacculus. In the majority of dsDNA phages muralytic enzymes are known to be a structural part of the virion (Moak and Molineux, 2004). Since the bacterial cell must remain metabolically active and maintain cell envelope integrity the action of peptidoglycan-hydrolyzing proteins must be well controlled and cause only local degradation of the peptidoglycan. Based on substrate specificities muralytic enzymes are classified into four groups: N-acetyl-muramidases, N-acetylglucosamidases, N-acetyl-muramoyl-L-alanine amidases and endopeptidases (see Fig. 1C).

Membrane-containing bacteriophages belonging to the *Cystoviridae*, *Tectiviridae* and *Corticoviridae* families have been shown to contain peptidoglycan-hydrolyzing activities associated with the virion. In the case of enveloped dsRNA cystovirus ϕ6, the lytic endopeptidase, protein P5, is associated with the nucleocapsid surface. It is active against the cell walls of several gram-negative bacteria, but shows no activity against gram-positive species. It was determined that the enzyme cleaves the cell wall peptide bridge formed by meso-2,6-diaminopimelic acid and D-alanine (Caldentey and Bamford, 1992). Tectivirus PRD1 virion contains two structural proteins possessing muralytic activity (P7 and P15). However, muramidase P15 was shown to be non-essential for entry and only transglycosylase P7 plays an crucial role in genome penetration (Rydman and Bamford, 2000). Inner membrane-containing corticovirus PM2 also contains a virion-associated muralytic activity. Viral structural protein P7 was proposed to degrade the peptidoglycan layer during the PM2 entry (Kivelä et al., 2004). Interestingly, zymogram analysis of tailless ssDNA bacteriophages G4 and ϕX174 did not indicate any muralytic activity associated with the virion (Moak and Molineux, 2004).

In addition, a study by Moak and Molineux demonstrated muralytic activities associated with the virions of an array of bacteriophages infecting Gram-negative and Gram-positive hosts (Moak and Molineux, 2004). It was proposed that peptidoglycan-degrading enzymes may be common among tailed bacteriophages. Muralytic activity associated with the tailed bacteriophage virions resides either within the tail structure (for myoviruses and siphoviruses) or within an internal head protein that forms part of the extensible tail (for podoviruses; (Moak and Molineux, 2004; Molineux, 2001). Peptidoglycan-hydrolyzing enzymes of bacteriophages T3 and ϕYeO3-12, closely related to phage T7 protein gp16, make genome penetration more efficient but are dispensable under optimal growth conditions (Moak and Molineux, 2000; Moak and Molineux, 2004). The baseplate structure of the tail of bacteriophage T4 contains protein gp5, which has an *N*-acetylmuramidase activity (Nakagawa et al., 1985).

Interestingly, protein Pb2 of bacteriophage T5 was shown to possess multiple functions in virion assembly and genome penetration; in addition to acting as a tail tape measure protein it possesses fusogenic as well as muralytic activity (Boulanger et al., 2008). Similar multi-functionality is characteristic to the terminal protein gp3 of bacteriophage ϕ29, which not only plays a role in phage DNA replication and packaging but was also shown to contain the muralytic activity (Meijer et al., 2001; Moak and Molineux, 2004). Structural protein P17 of *Staphylococcus aureus* phage P68 has also been shown to carry two activities – receptor binding and muralytic ones (Takac and Blasi, 2005).

2.3.2 Genome injection through a vertex

The majority of bacteriophages deliver their genome through one of the vertices. This strategy is employed by all tailed bacteriophages (order Caudovirales) and icosahedral phages belonging to the *Tectiviridae*, *Microviridae* and *Leviviridae* families.

Bacteriophage T4 (family *Myoviridae*) virion consists of a head and a long, contractile tail that ends with a hexagonal baseplate and tail fibers. After the binding to the host, the virion of T4 undergoes major structural rearrangements that cause the sheath around the tail tube to contract (Moody, 1973). The contraction of the outer sheath makes the tail tube to protrude from beneath the baseplate. A needle-like structure composed of gp27 and gp5 and located under the baseplate has been proposed to initiate the penetration through the cell envelope (Kanamaru et al., 2002). Gp5 locally degrades the peptidoglycan layer allowing the tail tube to penetrate (Nakagawa et al., 1985). The detailed mechanism of how the linear 172 kbp long dsDNA genome crosses the CM of the host in 30 seconds is not known. It was shown that entering T4 induces channel formation for genome delivery into the host cell. The insertion of the channel was shown to be dependent on the membrane voltage across the CM (Boulanger and Letellier, 1988). Interestingly, it was recently demonstrated that bacterial type VI secretion apparatus is structurally very similar to bacteriophage tails both long contractile and long non-contractile. Three components of type VI secretion system were shown to be homologous to bacteriophage tail proteins (Leiman et al., 2009; Pell et al., 2009). A plausible model for T4 DNA entry into the cell was proposed. The tail tube penetrates through the OM and the peptidoglycan layer, reaching the CM. The interaction of the tail tube with the CM results in phage genome injection. The cylinder formed by gp27 serves as a channel for DNA translocation (Kanamaru et al., 2002). It was also suggested that phage T4 induces transient fusion of the outer and cytoplasmic membranes allowing the genome to be delivered into the cell interior through a channel formed by the tail tube (Tarahovsky et al., 1991).

Siphovirus T5 possesses a long and non-contractile tail. Binding of bacteriophage to its receptor FhuA, results in conformational changes in protein Pb2 subsequently leading to its insertion into the host cell envelope (Feucht et al., 1990). By a yet unknown mechanism the head-connector opening is triggered and dsDNA genome of T5 is released from the head. Protein Pb2 bearing peptidoglycan degrading and fusogenic activities presumably forms a channel across the cell envelope for genome penetration (Boulanger et al., 2008; Guihard et al., 1992). The genome transfer is accomplished in two steps: first, 8% of the DNA is transferred to the cytoplasm. Then, there is a four minutes pause during which two proteins (A1 and A2) encoded by the leading DNA fragment are synthesized. The transfer of the remaining DNA is dependent on proteins A1 and A2 (Letellier et al., 1999).

In the case of short-tailed podovirus ϕ29, infecting gram-positive *Bacillus subtilis* cells, delivery of the linear genome with terminally attached proteins seems to occur in a manner similar to that of T5 (Gonzalez-Huici et al., 2004). In the first step ~60% of the phage DNA is pushed into the cell most probably by the pressure built inside the viral capsid. The remaining 40% of the genome are pulled from inside the cell by energy-requiring molecular machinery. Early viral protein p17 was proposed to play a central role during the second step of genome penetration, although other viral proteins seem to be also required (Gonzalez-Huici et al., 2004).

Bacteriophage T7 (family *Podoviridae*) has a short non-contractile tail, which is too short to span the cell envelope of its gram-negative host cell. Nevertheless, in order to get the T7 genome into the cell interior, such channel for genome delivery has to be formed across the cell envelope. The exact mechanism has not yet been fully understood. However, it was demonstrated that the channel is formed by internal core proteins gp14 and gp16 that are ejected from the phage capsid (Chang et al., 2010; Garcia and Molineux, 1996; Molineux, 2001). During the initiation of T7 infection the tail protein gp7.3 and the head protein gp6.7 leave the virion followed by injection of the other three above mentioned phage proteins. Once the channel is formed, penetration of the T7 DNA into the host cell occurs. The T7 genome delivery into the host is a multistep enzyme-catalysed, energy-requiring process (Kemp et al., 2004). First, only about 850 base pairs of the genome are ejected. The leading part of the DNA contains three early promoters A1, A2, and A3 recognized by E. *coli* RNA polymerase (Garcia and Molineux, 1995; Garcia and Molineux, 1996). Consequently, approximately 7 kb of the genome are drawn into the cytoplasm as a result of transcription by the host RNA polymerase. The T7 RNA polymerase, which is synthesized from these early transcripts, then recognizes phage promoters and pulls the remaining part of the genome into the cell (Garcia and Molineux, 1996). This multistep translocation of the entire bacteriophage T7 genome into the host cytoplasm takes between 9 and 12 minutes (Garcia and Molineux, 1995).

The icosahedral dsDNA bacteriophage PRD1 of the *Tectiviridae* family has an internal lipid membrane between the genome and the capsid. Electron microscopy (EM) pictures indicate empty virus particles associated with the host cell. It was therefore suggested that this tailless bacteriophage uses the same strategy as employed by tailed phages - genome delivery through the vertex (Grahn et al., 2002). Virion interaction with the receptor induces structural rearrangements of the vertex structure. The spike complex is removed from the vertex resulting in a formation of an opening with a diameter of 14 nm (Butcher et al., 1995; Rydman et al., 1999). Virus encoded protein P11 was shown to be essential for interaction with the host OM and protein P7 is required for local degradation of the peptidoglycan layer. Subsequently, phage membrane transforms into a tubular structure and crosses the cell envelope. The translocation of the PRD1 genome is dependent on viral integral membrane proteins P14, P18 and P32 (Grahn et al., 2002). It was proposed that part of the energy required for the genome delivery is conserved during genome packaging. A transformation of the protein-rich viral membrane into a lower energy state (i.e. tubular structure) might also play a role in PRD1 genome entry into the host cytosol (Grahn et al., 2002).

Icosahedral ssDNA bacteriophages of the *Microviridae* family also deliver their genomes into the host through a vertex, leaving the empty capsid associated with the infected cell surface. The best studied member of this family is ϕX174. Phage binding to the cellular receptor triggers structural rearrangements in the spike protein G and subsequent formation of an opening in the capsid. This opening serves as a channel for DNA delivery. EM pictures show ϕX174 particles embedded in the cell envelope at the points of adhesion zones, where the outer and cytoplasmic membranes are close to each other (Bayer and Starkey, 1972; Brown et al., 1971). Initially, the pilot protein H and the incoming genome are found in association with the OM at the adhesion zones. Then, the ssDNA together with the pilot protein is delivered into the cell interior and remains associated with the CM, the site of DNA replication (Jazwinski et al., 1975a).

Not much is known about the entry mechanism of ssRNA phages (family *Leviviridae*). Nevertheless, they were

shown to deliver their genome into the host through one vertex of the icosahedral capsid. After the adsorption the receptor binding protein is cleaved into two fragments resulting in the opening of a fivefold vertex through which the genome exits the capsid. How the ssRNA genome enters the host cytoplasm is not known. It is possible that leviviruses exploit their receptor, the pilus, to maneuver the genome into the host, leaving the capsid on the outside (Van Duin and Tsareva, 2006).

2.3.3 Capsid dissociation at the cell envelope

This strategy of entry is employed by filamentous bacteriophages (family *Inoviridae*) and corticovirus PM2. The best studied is the entry mechanism of filamentous Ff phages (the closely related M13, f1 and fd). It is presumed that other filamentous bacteriophages penetrate the host envelope in a similar manner. The minor virion protein pIII, that is located at one end of the helical phage particle, mediates phage binding to the receptor and is also required for uncoating of the virion and subsequent genome penetration into the host cytoplasm (Endemann et al., 1992; Endemann and Model, 1995). The host protein complex TolRQA, spanning the CM and extending into the periplasm was found to be essential for viral DNA penetration (Click and Webster, 1998).

As mentioned above, interaction of the pIII N2 domain with the pilus receptor results in retraction of the latter. The exact mechanism of how the virion crosses the OM of the host is unknown, but it is suggested that the pilus might pull the virion into the periplasm (Click and Webster, 1997). The interaction of the N2 domain with the receptor makes pIII domain N1 available for binding to the integral cytoplasmic membrane protein TolA (Riechmann and Holliger, 1997). Upon this interaction conformation of the C-terminal domain of pIII changes leading to the exposure of the hydrophobic anchor of pIII, which in turn inserts into the cytoplasmic membrane, thereby anchoring the virion. This leads to ”unlocking” of the virion and adjacent major coat proteins are being inserted into the CM. As a result, the phage ssDNA is released (Bennett and Rakonjac, 2006). Translocation of the phage genome into the host cytoplasm might be coupled to the insertion of the capsid proteins into the CM (Click and Webster, 1998). How the ssDNA genome of the phage penetrates through the CM is not known. It was proposed that a channel for DNA delivery could be formed directly by pIII or the transmembrane helixes of the cytoplasmic membrane protein complex TolQRA or a combination of both (Bennett and Rakonjac, 2006; Click and Webster, 1998; Riechmann and Holliger, 1997).

The protein coat of marine bacteriophage PM2 that infects gram-negative bacteria also dissociates at the host cell envelope. After the binding of the virion to the receptor, no empty particles bound to the host cells were observed. Instead, proteins P1 and P2 composing the capsid of the virion were found to be released into the medium upon genome internalization. Disassembly of the PM2 coat exposes the fusion-competent lipid core. It was proposed that the viral membrane fuses with the OM of the host and the genome is released into the periplasmic space (Kivelä et al., 2004). Most probably only the PM2 genome enters the cytoplasm as electroporation of naked circular PM2 genome into the host cells resulted in normal virus replication (Krupovič et al., 2006), however the actual mechanism for genome entry remains unknown.

2.3.4 Fusion and endocytosis-like penetration

The enveloped dsRNA bacteriophage ϕ6 (*Cystoviridae* family) enters the host in a unique manner, which resembles mechanisms utilized by eukaryotic, rather than bacterial, viruses. After adsorption to the receptor, the virion gets into a close contact with the host OM. The viral transmembrane protein P6 mediates the fusion between the virion envelope and the bacterial OM. The virus-encoded lytic enzyme locally hydrolyzes the peptidoglycan layer and the nucleocapsid reaches the CM. The nucleocapsid penetrates across the CM in an endocytosis-like event, which is mediated by the nucleocapsid shell protein P8 (Bamford et al., 1987; Poranen et al., 1999). For more detailed ϕ6 entry description see section 3.1.1.

Bacteriophages of the *Plasmaviridae* family are enveloped, quasi-spherical particles infecting mycoplasmas, parasitic bacteria that lack the cell wall and possess only the cytoplasmic membrane. It is assumed that adsorption of pleomorphic bacteriophage L2 to the receptor leads to the fusion of phage and host cell membranes, leading to delivery of the virion nucleoprotein into the host cytoplasm (Maniloff and Dybvig, 2006).

3. Bacteriophages used in this study

3.1 Family *Cystoviridae*

Bacteriophages belonging to the family *Cystoviridae* share a common overall virion organization. They contain a lipid envelope and a segmented genome comprised of three double-stranded RNA molecules. Cystoviruses are the only characterized bacteriophages that possess a dsRNA genome (Qiao et al., 2000). The isolated cystoviruses could be divided into two major groups: (i) those closely related to the type member of the family ϕ6 (ϕ7, ϕ9, ϕ10, ϕ11 and ϕ14) and (ii) those distantly related to ϕ6 (ϕ8, ϕ12, ϕ13, ϕ2954; Gottlieb et al., 2002; Mindich et al., 1999; Qiao et al., 2010).

3.1.1 Bacteriophage ϕ6

ϕ6 is the first isolated member of the *Cystoviridae* and is consequently the best-studied phage in this virus family. Bacteriophage ϕ6 was isolated in 1973 from the gram-negative bacterium *Pseudomonas syringae* pathovar *phaseolicola* (Vidaver et al., 1973)

Virion. The spherical virion contains three structural layers: (i) the external lipid membrane, (ii) the nucleocapsid (NC), and (iii) the inner core particle also known as the polymerase complex (Bamford and Mindich, 1980). The genome, consisting of three linear segments of dsRNA, resides inside the core (Semancik et al., 1973). The schematic representation of the virion structure is depicted in Figure 3. The virion has 25 % lipids, 13 % RNA, and 62 % proteins (Vidaver et al., 1973). The determined diameters are: 86 nm for the virion, 58 nm - the NC, and 50 nm - the core (Butcher et al., 1997; Kenney et al., 1992).

The bacteriophage envelope is composed of lipids derived from the host cytoplasmic membrane and five virus-encoded proteins: P3, P6, P9, P10 and P13 (Laurinavičius et al., 2004a; Sands, 1973; Sinclair et al., 1975). The phospholipid analysis revealed that ϕ6 envelope is

composed of 58 % PE, 38 % PG, and 4 % CL, making the overall phospholipid class composition of ϕ6 more similar to that of CM than OM of the host (Laurinavičius et al., 2004a). It was suggested that phospholipids from the host cytoplasmic membrane are not randomly incorporated into the ϕ6 envelope, but the process is selective. The selection could be based on (i) preferential interaction of integral membrane proteins with certain phospholipid molecular species or (ii) optimal packing of phospholipid molecules in the viral membrane (Laurinavičius et al., 2004a). Four envelope proteins (P6, P9, P10, and P13) are spanning the viral membrane, while the fifth envelope protein, P3, is attached to protein P6 and forms the spikes that protrude from the virion surface (Kenney et al., 1992; Mindich et al., 1976).

The nucleocapsid surface shell is made of protein P8 (Day and Mindich, 1980). P8 is arranged on a T=13 lattice with exceptions around the fivefold symmetry axes that are occupied by hexameric protein P4 (Butcher et al., 1997). The stability of the P8 shell is dependent on Ca^{2+} ions (Olkkonen and Bamford, 1987). Protein P5, the lytic enzyme, is loosely associated with the NC surface and resides between P8 layer and the viral envelope (Hantula and Bamford, 1988).

The innermost layer of ϕ6 virion, the viral core, is composed of four proteins P1, P2, P4, and P7 (Bamford and Mindich, 1980; Mindich and Davidoff-Abelson, 1980). Dimers of protein P1 arranged on a T=1 icosahedral lattice form the skeleton of the core (Olkkonen and Bamford, 1987). Hexameric packaging NTPase P4 forms turret-like protrusions on the P1 lattice and occupies the fivefold positions of the P1 cage (Butcher et al., 1997; de Haas et al., 1999). P2, an RNA-dependent RNA polymerase, occupies positions closer to adjacent 5-fold vertices where it conducts replication and transcription (Makeyev and Bamford, 2000; Sen et al., 2008) Minor polymerase core protein P7 is an assembly factor. The exact location of P7 within the ϕ6 polymerase complex is currently unknown (Poranen et al., 2008).

Genome. The genome of the ϕ6 is composed of three dsRNA segments (L, M, and S; Semancik et al., 1973). Every virion contains one copy of each segment (Day and Mindich, 1980). The exact sizes are 6374 bp, 4063 bp, and 2948 bp for L, M, and S, respectively (Gottlieb et al., 1988; Mindich et al., 1988). The L segment encodes the early-expressed proteins that assemble into the polymerase complex (P1, P2, P4, and P7) and nonstructural protein P14 (Casini and Revel, 1994; Mindich et al., 1988). The M segment codes for the envelope proteins P3, P6, P10 and P13, while the S encodes NC shell protein P8, major membrane protein P9, proteins responsible for host cell lysis P5 and P11, and nonstructural protein P12 (Gottlieb et al., 1988; McGraw et al., 1986). The coding regions of all three segments are flanked by distinct noncoding regions, containing essential signals for genome packaging and replication (Gottlieb et al., 1988; McGraw et al., 1986; Mindich et al., 1988).

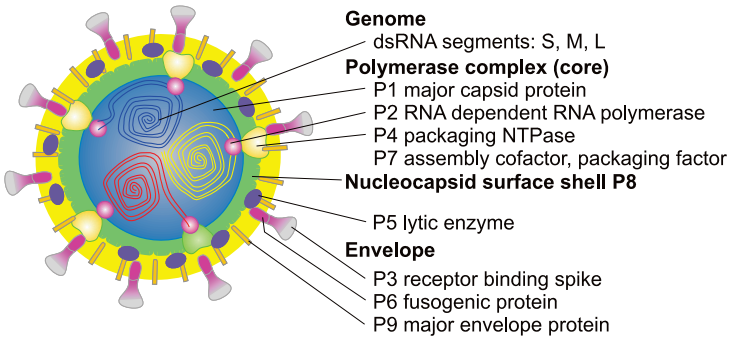


Figure 3. Schematic presentation of the bacteriophage ϕ6 virion. The ϕ6 particle consists of a procapsid composed of proteins P1, P2, P4, and P7, as well as the three segments of the dsRNA viral genome. The procapsid and a shell of proteins P8 form the nucleocapsid. A lipid membrane containing membrane proteins P3, P6, P9, P10 and P13 form the outermost layer of the particle. The picture is courtesy of Dr. Minna Poranen.

Life cycle. Bacteriophage ϕ6 genome is retained within the viral polymerase complex during the entire viral life cycle. Therefore, the mechanism of ϕ6 entry into the host cell is distinct from that of other bacteriophages and resembles the route utilized by animal viruses. The receptor for ϕ6 on the host cell surface is a chromosomally encoded type IV pilus (Bamford et al., 1976; Roine et al., 1998; Vidaver et al., 1973). *P. syringae* are plant pathogens and use these pili to adsorb to the leaf surface of the host plant. Bacteriophage ϕ6 attaches to the side of the pilus with its spike protein P3. The subsequent retraction of the pilus pulls the phage through the lipopolysaccharide layer and brings it into close proximity of the bacterial OM (Romantschuk and Bamford, 1985; Romantschuk and Bamford, 1986). The receptor-binding spike protein P3 is then removed and virus encoded protein P6 mediates fusion of viral envelope with the host OM. After the fusion, NC is released into the periplasmic space without leakage of the periplasmic content into the extracellular milieu (Bamford et al., 1987). The NC-associated endopeptidase P5 locally digests the peptidoglycan layer by cleaving the peptide bond between meso-diaminopimelic acid and D-alanine. As a result, the NC reaches the host CM (Mindich and Lehman, 1979).

Based on the EM data, penetration of the NC particle into the cytosol takes place via membrane invagination and formation of the intracellular membrane vesicle enclosing the NC. Such process is reminiscent of the endocytotic entry of animal viruses (Romantschuk et al., 1988). The penetration of the NC into the cytosol is membrane voltage dependent. Nucleocapsid surface protein P8 was shown to be crucial for the interaction between the NC and the CM (Ojala et al., 1990; Olkkonen et al., 1990; Poranen et al., 1999). It was demonstrated that interaction between the NC protein P8 and the phospholipids of the host CM is essential and sufficient for internalization of the NC (Cvirkaitė-Krupovič et al., 2010). However, the exact mechanisms of how the NC is finally released from the endocytic-like vesicle into the cytosol and how it is uncoated are not known. However, it was suggested that pH-dependant events are crucial during the NC release from the entry vesicle (Poranen et al., 1999). After the NC enters the host cytoplasm P8 shell dissociates leading to the activation of the polymerase complex.

The genome-containing polymerase complex produces the single-stranded copies [small (s), medium (m), and large

(l)] of the three genomic segments that serve as mRNAs for the production of viral proteins and are also packaged into the newly synthesized virions. All three mRNAs are produced early in the infection, but only l mRNA is translated at 10 min post infection to produce proteins P1, P2, P4, and P7 that assemble into the procapsids (PC; Bamford and Mindich, 1980). The procapsids package the three plus-strand RNAs. The packaging is dependent on the NTP hydrolysis by viral NTPase P4 (Frilander and Bamford, 1995; Gottlieb et al., 1991). Packaging involves the recognition of the specific sequences present at the 5' termini of the ssRNA molecules and continues until all three segments are packaged. The three plus-strand RNAs have different binding affinities to the PC (s > m > l; Juuti and Bamford, 1995; Mindich, 1999), and enhance each other's packaging in the same order (Frilander and Bamford, 1995; Qiao et al., 1995). These differences ensure that the progeny virions contain all three segments of the genome. The packaged plus-sense RNAs are replicated into the dsRNAs by viral polymerase P2 within the core particle (Frilander et al., 1992). The assembly of P8 shell onto the core particle turns off the transcription (Olkkonen et al., 1991). Then nucleocapsids acquire the membrane, together with the envelope proteins (Bamford et al., 1976). The envelopment of ϕ6 does not involve budding from the host cytoplasmic membrane but rather preformed lipid vesicles envelope the NCs in the cell interior by an unknown mechanism (Stitt and Mindich, 1983).

The first enveloped particles appear at about 45 min post infection and are localized in the center of the infected cell, without any apparent association with the CM (Bamford et al., 1976). The virion maturation is completed by the addition of the spike protein P3 onto the virion (Mindich and Lehman, 1979). The release of new virions from the cell is dependent on the lytic protein P5 and the integral membrane protein P11. Upon cell lysis (~90 min post infection) ~150-200 progeny phage particles are liberated per one infected cell (Vidaver et al., 1973).

3.1.2 Bacteriophage ϕ13

Virion. Bacteriophage ϕ13 was isolated from the leaves of the radish plant (*Raphanus sativum*; Mindich et al., 1999). Based on the cryo-electron microscopy data, the overall organization and size of the ϕ13 virion is very similar to that of bacteriophage ϕ6; dsRNA-filled cores are covered with the protein P8 shell and have a protein rich phospholipid membrane with protruding spikes. The diameters of the ϕ13 virion and NC are ~85 and ~58 nm, respectively (Yang et al., 2003).

The proteins making the polymerase complex (proteins P1, P2, P4, and P7) of ϕ13 are significantly similar at the sequence level to the corresponding proteins of ϕ6. However, the proteins P6, P8 and P12 display only moderate similarity (~ 22%) to their homologues in ϕ6. Although the overall virion organization is similar to that of ϕ6, the host attachment protein P3 is a dimer in ϕ13 (Mindich et al., 1999).

Genome. The genome of bacteriophage ϕ13 is also tripartite dsRNA. The sizes of the three segments were found to be 6458, 4213, and 2981 bp for L, M, and S segments, respectively (Qiao et al., 2000). The organization of the genes in segments L, M and S is similar to that found in equivalent segments of ϕ6. Genes in segments M and S were named after those found in the same position in ϕ6 (Mindich et al., 1999; Qiao et al., 2000).

As in bacteriophage ϕ6, the coding regions of all three ϕ13 genomic segments are flanked by noncoding regions. At the 5'

ends the three segments there is a similar region of 11 bases (*pac* region) that is essential for packaging of the ssRNA molecules. In contrast, the sequences at the 3' ends of the segments are very different from each other at the nucleotide level, but were predicted to adopt similar secondary structures. The 3' regions of the genome are not involved in packaging specificity, but are important for polymerase recognition.

Life cycle. The knowledge about ϕ13 life cycle is poor. It was proposed that ϕ13 uses different receptor for adsorption to the host cell than ϕ6. It attaches directly to rough LPS or some element exposed on the surface but not to the type IV pilus as ϕ6 does (Mindich et al., 1999).

3.2 Family *Tectiviridae*

Phages belonging to the *Tectiviridae* (from the Latin *tectus* meaning covered) virus family possess a linear dsDNA genome and a membrane bilayer beneath the icosahedral protein shell. These bacteriophages are divided into two groups: those infecting gram-negative and gram-positive bacteria. The group infecting gram-negative hosts includes bacteriophage PRD1 (the type member of the family) and five closely related phages: PR3 (Bradley and Rutherford, 1975), PR4 (Stanisich, 1974), PR5 (Wong and Bryan, 1978), PR772 (Coetzee and Bekker, 1979), and L17. Five members infecting gram-positive bacteria have been reported so far: Bam35 (Ackermann et al., 1978), AP50 (Nagy and Ivanovics, 1977), phiNS11 (Bamford and Ackermann, 2000), GIL01 and GIL16 (Verheust et al., 2005; Verheust et al., 2003).

Bacteriophage PRD1 is the best studied member of the *Tectiviridae* family. It is an icosahedral, tailless, inner membrane-containing bacteriophage (Olsen et al., 1974). Underneath the protein capsid resides the membrane enclosing a linear, ~15 kbp long dsDNA genome with terminal proteins covalently attached to 5' ends (Davis et al., 1982). The major coat protein P3 is organized on the T=25 lattice (Abrescia et al., 2004; Butcher et al., 1995). P31 pentamers occupy the vertices and form a base of the vertex complex, which is composed of the spike protein P5 and the receptor binding protein P2 (Huiskonen et al., 2007; Rydman et al., 1999).

PRD1 infects a broad range of gram-negative bacteria carrying conjugative multi-drug resistance plasmids of the IncP-, IncN- or IncW type. Mating pair formation (Mpf) complex serves as a receptor for PRD1 (Grahn et al., 1997). Interaction of protein P2 with the receptor results in a conformational change and removal of P2 from the virion, followed by release of the rest of the vertex proteins and the peripentonal capsid protein trimers (Grahn et al., 1999). After the removal of the vertex complex, the spherical inner membrane vesicle is transformed into a tube-like structure, which is used as a channel for genome delivery into the host (Bamford and Mindich, 1982; Grahn et al., 2002). Phage structural proteins P11, P14, P18 and P32 are involved in genome delivery (Grahn et al., 2002), while virion-associated muralytic enzyme P7 locally digests the peptidoglycan layer (Grahn et al., 2002; Rydman and Bamford, 2000).

The PRD1 genome is replicated by a protein-primed mechanism (Caldentey and Bamford, 1992; Caldentey et al., 1993). Once the empty membrane-containing procapsids are assembled inside the cell, they are packaged with the phage DNA by the genome packaging ATPase P9 (Strömsten et al., 2005). At the end of the PRD1 infection cycle the host cell is lysed as a result of the concerted action of phage-encoded holin P35, endolysin P15, and a pair of accessory lysis proteins P36 and P37

(Krupovič et al., 2008; Rydman and Bamford, 2003; Žiedaitė et al., 2005).

3.2.1 Bacteriophage Bam35

Virion. The temperate bacteriophage Bam35 was initially isolated from *Bacillus thuringiensis* sv. alesti strain 35 (Ackermann et al., 1978). As determined by negative-stain electron microscopy (Ackermann et al., 1978; Ravantti et al., 2003) and recently confirmed by electron cryo-microscopy and three-dimensional image reconstruction (Laurinmäki et al., 2005) the morphology of Bam35 virion closely resembles that of PRD1. Bam35 virion has an isometric protein coat surrounding the lipid bilayer, which encloses the linear dsDNA genome with covalently linked terminal proteins (Fig. 4; Ackermann et al., 1978; Laurinavičius et al., 2004b; Ravantti et al., 2003). About 15 nm long spikes protrude from the vertices of the virion. The diameter of the whole virion, between opposite vertices is ~73 nm (Laurinmäki et al., 2005). The viral membrane is derived from the host cytoplasmic membrane. The phospholipid analysis revealed that the viral membrane is composed of ~30 % PE, ~53 % PG and ~17 % CL. Such composition is different from that of the host (~57 % PE, ~30 % PG and ~13 % CL), suggesting that lipids are selectively incorporated into the virion during the assembly (Laurinavičius et al., 2004b).

The most abundant protein in Bam35 virion is the major coat protein gp18. The trimers of gp18 are organized on the T=25 icosahedral lattice. Cryo-EM reconstruction showed that the fold of Bam35 major capsid protein is similar to that of PRD1 (Laurinmäki et al., 2005). Based on genomic location gene product of ORF27 was predicted to be the penton protein, while protein complexes at the vertexes are suggested to be composed of gp28 and gp29. Based on size and proline content several candidates for the Bam35 tape measure protein, a protein dictating the distance between the icosahedral vertexes, were suggested (Laurinmäki et al., 2005; Ravantti et al., 2003).

Genome. The genome of bacteriophage Bam35 is a linear, 14 935-bp-long dsDNA molecule, and contains covalently linked terminal proteins at the 5' ends. An average GC content was found to be 39,7 %. Inverted terminal repeats are 74 bp long and display 81% identity. The organization of the Bam35 genome closely resembles that of PRD1, although there is no detectable sequence similarity (Ravantti et al., 2003). The Bam35 genome shares high sequence similarity with *B. thuringiensis* phages GIL01 and GIL16 and the linear *Bacillus cereus* plasmid pBClin15 (Strömsten et al., 2003; Verheust et al., 2005; Verheust et al., 2003).

Using computational methods 32 open reading frames (ORF) longer than 100 bp were predicted. All of them were on the same DNA strand. The majority of Bam35 ORFs overlap confirming that phage genome is tightly packed. The putative genes coding for Bam35 DNA polymerase (ORF5), LexA-type DNA-binding protein (ORF6), genome packaging ATPase (ORF14), peptidoglycan hydrolyzing proteins (ORF26 and ORF30) have been identified (Ravantti et al., 2003).

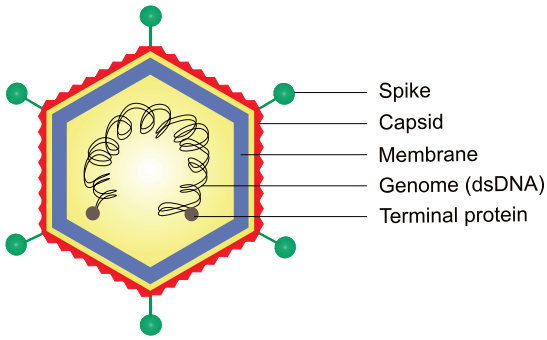


Figure 4. Schematic presentation of the bacteriophage Bam35 virion. The genome of Bam35 is enclosed within a membrane vesicle, which is surrounded by an icosahedral capsid shell.

Life cycle. Bacteriophage Bam35 can either establish a carrier state and reside inside the cell as a linear plasmid, i.e., lysogenize its host, or lyse the infected cells leading to the release the progeny into the growth medium (Gaidelyte et al., 2005; Strömsten et al., 2003). It is assumed that Bam35, like gram-negative hosts-infecting tectivirus PRD1, uses the tube-like membrane structure to deliver the genome into the cytosol through the thick peptidoglycan layer and the CM (Laurinmäki et al., 2005; Ravantti et al., 2003). When the genome is delivered into the cell, the empty virus particles are tightly attached to the cell surface. Based on electron microscopic data, at about 40 minutes post infection mature virus particles start accumulating in the center of the infected bacterial cell. Cell lysis begins ~45 minutes post infection and is complete in fifteen minutes (Ravantti et al., 2003). Energy poisons added to Bam35 infected cells were able to induce premature lysis, pointing to the possibility that virus employs the holin-endolysin system to disrupt the host cells at the end of the infection cycle (Gaidelyte et al., 2005).

3.3 Family *Corticoviridae*

3.3.1 Bacteriophage PM2

The marine bacteriophage PM2 is the only isolated member of the *Corticoviridae* family, but PM2-like prophages commonly reside in the genomes of aquatic bacteria (Bamford and Bamford, 2006; Krupovič and Bamford, 2007). PM2 was isolated from the costal sea waters of Chile (Espejo and Canelo, 1968b). It infects gram-negative marine bacteria of the genus *Pseudoalteromonas* (Espejo and Canelo, 1968a; Kivelä et al., 1999).

Virion. PM2 is an icosahedral inner membrane-containing bacteriophage (Fig. 5). The circular dsDNA genome is enclosed within a protein-rich membrane vesicle (Kivelä et al., 2002). The genome and the inner membrane were designated as the lipid core (LC; Kivelä et al., 2002). LC is covered with an icosahedral protein shell. Virion mass is estimated to be approximately 44 MDa (Abrescia et al., 2008), where proteins comprise ~72 %, nucleic acid, ~14 % and lipids ~14 % (Camerini-Otero and Franklin, 1975). Using cryo-EM and three-dimensional image reconstruction structure of PM2 was solved to a resolution of 8.4 Å (Huiskonen et al., 2004). The entire PM2 virion has also been recently crystallized and structure solved by

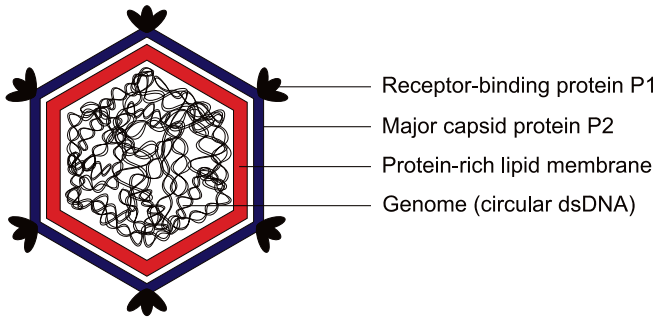


Figure 5. Schematic presentation of the bacteriophage PM2 virion. The genome of PM2 is enclosed within a membrane vesicle, which is surrounded by an icosahedral capsid shell.

X-ray crystallography at 7 Å resolution. The high resolution structures of spike protein and the major capsid protein are available (Abrescia et al., 2008). The overall PM2 capsid dimensions are as follows: facet-to-facet - 56.5 nm, edge-to-edge - 59.0 nm, vertex-to-vertex - 63.5 nm, spike-to-spike - 76.5 nm (Huiskonen et al., 2004).

Ten virus-encoded proteins (P1 – P10) have been identified as structural components of the PM2 virion (Huiskonen et al., 2004; Kivelä et al., 2002; Kivelä et al., 1999). The icosahedral protein coat is composed of the major capsid protein P2, organized on a T= 21 lattice, and the pentameric receptor-binding protein P1, which occupies the vertices of the icosahedral capsid (Abrescia et al., 2008; Kivelä et al., 2002). All the other proteins (P3 – P10) are associated with the LC (Huiskonen et al., 2004; Kivelä et al., 2002; Kivelä et al., 1999).

Lipids are selectively incorporated from the host CM during the virion assembly (Braunstein and Franklin, 1971). The lipid composition of the PM2 membrane is ~64 % PG, ~27 % PE, and ~8% neutral lipids and small amount of acyl-PG, while the host CM is mainly composed of ~75 % PE and ~25 % PG (Camerini-Otero and Franklin, 1972; Tsukagoshi et al., 1976). The membrane in the virion follows the shape of the capsid (Huiskonen et al., 2004).

Genome. The PM2 genome is a highly supercoiled circular dsDNA molecule (Espejo et al., 1969). PM2 DNA has the highest number of negative supercoils (51) detected in a naturally occurring molecule (Gray et al., 1971). The sequence of the entire PM2 genome revealed that it is a 10 079 bp long molecule (Männistö et al., 1999).

The phage genome is organized into three operons – two early and one late. OEL is a leftward transcribing early operon which is under control of promoter P_{1207}. It was shown to code for two transcriptional repressors (P15 and P16). P16 negatively controls the early right operon (OER, under control of promoter P_{1193}), while protein P15 in addition to the OER, represses its own promoter. Expression of the late operon (OL - under control of P_{5321}), that codes for almost all structural PM2 proteins (P9 is coded in OER) as well as the two lysis proteins is positively controlled by proteins P13 and P14 (Männistö et al., 2003).

Life cycle. PM2 infection starts with the recognition of the phage receptor on the host cell surface via pentameric receptor-binding protein P1 (Huiskonen et al., 2004; Kivelä et al., 1999). In spite of considerable efforts the receptor for PM2 remains unidentified. Neither the LPS isolated from PM2-sensitive cells nor different cellular fractions (soluble and insoluble) interfered with PM2 adsorption. Moreover, extensive protease treatment of the host cells did not affect PM2 binding (Kivelä et al., 2004). High resolution X-ray structure of protein P1 indicates that Ca^{2+} ion is bound in a cleft at the tip of the protein. Systematic comparison revealed that P1 is structurally

most similar to a Ca^{2+}-dependent carbohydrate-binding protein from a marine bacterium *Saccharophagus degradans*. The Ca^{2+} ion in P1 is in a similar position as in bacterial protein, where it marks the site of sugar binding (Abrescia et al., 2008). Accordingly, it was proposed that LPS might serve as a receptor for PM2 (Kivelä et al., 2008). After the adsorption to the cell surface the protein capsid composed of proteins P1 and P2 dissociates and fusion-competent LC is exposed to the OM of the host (Kivelä et al., 2004). Then the fusion of PM2 membrane with the host outer membrane takes place. It was suggested that virion-associated lytic enzyme locally degrades the peptidoglycan layer leading to the PM2 genome interaction with the CM (Kivelä et al., 2004). However, it is still not clear how PM2 circular supercoiled dsDNA penetrates through the CM.

As soon as the PM2 genome gets into the cytoplasm, its replication is initiated. The genome replication takes place in association with CM and proceeds via the rolling-circle mechanism; the EM data revealed PM2 replication intermediates consisting of double-stranded circular molecules with growing tails no longer than the length of the genome (Brewer, 1978b; Canelo et al., 1985; Espejo et al., 1971; Männistö et al., 1999). Replication starts when the phage-encoded initiation protein P12 nicks the DNA at the ORI site. The protein stays covalently bound to the 5' phosphate through a tyrosine residue present in its active site. The 3' end is extended by the DNA polymerase III and proceeds until the leading strand has been fully displaced (Khan, 2005). After 10 minutes post infections synthesis of the host DNA is inhibited, whereas PM2 dsDNA is being produced at a high rate throughout the entire infection cycle (Franklin et al., 1969). The viral genes are transcribed by the host DNA-dependent RNA polymerase (Bull et al., 1988; Zimmer and Millette, 1975a; Zimmer and Millette, 1975b).

The assembly of PM2 virions is likely to take place in association with the host cytoplasmic membrane. Two different virion assembly pathways have been proposed for PM2. According to the first proposed pathway the PM2 genome is packaged into the empty preformed procapsid, as seems to be the case for other internal membrane-containing dsDNA viruses (Brewer, 1978a; Strömsten et al., 2003). The alternative scenario, on the contrary, favors the co-assembly of viral genome and structural proteins into infectious virions (Abrescia et al., 2008). Whatever the assembly pathway is, the first intracellular particles are visible ~50 min post infection and are always lined-up along the host cytoplasmic membrane.

It was demonstrated that PM2 employs a lysis system not previously described for other bacteriophages (Krupovič et al., 2007a). A pair of PM2-encoded proteins P17 and P18 is necessary for successful disruption of the host envelope at the end of the infection cycle. Moreover, it was proposed that PM2 relies on a cellular lytic factor to degrade the peptidoglycan layer (Krupovič et al., 2007a). Approximately 60 minutes p.i. infected cells undergo lysis and approximately 300 new viral particles are released from one infected cell (Cota-Robles et al., 1968; Kivelä et al., 1999).

B. AIMS OF THIS STUDY

Viruses are biological entities that can reproduce only within the host cell. Therefore, entry into the host is a crucial step ensuring successful reproduction and survival of the virus. A viral genome contains all the information required for its replication in the cell. Consequently, viruses deliver the genome and accessory factors needed for replication into the host interior. However, entering viruses have to overcome the obstacle formed by the cell surface. In the case of bacteriophages, they have to cross a multilayered cell envelope of their hosts. The envelope of gram-positive bacteria consists of the cytoplasmic membrane and the external thick peptidoglycan layer, while gram-negative bacteria have one additional layer, the outer membrane. Viruses have devised numerous sophisticated strategies to overcome the cellular barriers and enter into their hosts in a well controlled manner.

The objects of this study are icosahedral, membrane-containing bacterial viruses belonging to three viral families – *Cystoviridae* (phages ϕ6 and ϕ13; Study I), *Tectiviridae* (phage Bam35; Study II), and *Corticoviridae* (phage PM2; Study III). The main goal was to elucidate and compare the entry mechanisms employed by these bacteriophages. The specific aims were as follows:

- To determine the characteristics of the phage binding to the host cell;
- To investigate the passage through the peptidoglycan layer;
- To study the phage entry-induced effects on the host cytoplasmic membrane;
- To evaluate the phage infection dependence on the energetic state of the cell;
- To examine the role of bacteriophage membrane during the entry process.

C. MATERIALS AND METHODS

Experimental procedures are summarized in Table 2 and described in the original publications. The references to the methods can be found from the articles.

Table 2. Methods used in this study

Method	Described and used in		
Determination of ATP content	I		
Dissociation of phage particles		II	
Electron microscopy	I		III
Fluorescence measurements	I		
Ion flux measurements	I	II	III
Isolation of peptidoglycan and removal of teichoic acids		II	
Labeling of phage particles	I		
Molecular cloning techniques		II	
One-step growth experiment	I	II	III
Phage adsorption assays	I	II	III
Phage growth and purification	I	II	III
Polyclonal antiserum production		II	
Protein purification		II	
Receptor saturation assay	I	II	
SDS polyacrylamide gel electrophoresis		II	
Zymogram analysis		II	

D. RESULTS AND DISCUSSION

1. Bacteriophage adsorption to the host cell

1.1 Receptor saturation

The first step in every virus infection is recognition of the correct host and binding to the specific receptor present on the cell surface. As mentioned above, virus capsid has a dual character – stability and lability. The lability of the capsid ensures the efficient delivery of the genome into the host cell. After binding to the cellular receptor major structural re-arrangements in the virion structure take place leading to delivery of the viral genome into the host.

To define the maximal number of virions bound per one host cell, receptor saturation assays were performed. It was determined that one *P. syringae* LM2489 cell could bind around 60 ϕ13 particles (I, Fig. 1C) and the receptor saturation was achieved at a multiplicity of infection (MOI) of ~100. The maximum number of bound bacteriophage PM2 particles was 180 per one ER72M2 cell, achieved at an MOI of ~300 (Kivelä et al., 2004). An even higher number of phage Bam35 virions could bind to the host cell surface. HER1410 cells bound ~500 Bam35 virions per one cell with signs of receptor saturation at an MOI of ~600 (II, Fig. 1C). These results indicate that molecules serving as receptors for these three bacteriophages are fairly abundant structure exposed on the surface of the corresponding host cells. For example, the number of LPS molecules, a receptor for ϕ13, per one bacterial cell is estimated to be $\sim 1 \times 10^6$ (Nikaido, 1996).

1.2 Effect of divalent cations on phage binding to the receptor

Divalent cations play an important role during different stages of bacteriophage life-cycle, and entry is no exception. For example, it was demonstrated that adsorption of some bacteriophages, such as *Bacillus* phage 4Ic (Landry and Zsigray, 1980), mycobacteriophage L5 (Fullner and Hatfull, 1997) is dependent on calcium ions.

Bacteriophage PM2 is a marine bacteriophage and its natural niche is sea water containing high concentrations of divalent cations (~10 mM Ca^{2+} and ~50 mM Mg^{2+}). We therefore tested if these divalent cations play a role in PM2 binding to the host. The adsorption test in the medium devoid of magnesium and calcium ions indicated that neither of these cations is required for phage binding to the receptor. On the contrary, when Ca^{2+} and Mg^{2+} were omitted from the infection medium, PM2 adsorption to the host was considerably faster, as could be judged from the higher adsorption rate constants (III, Fig. 2A). Divalent cations are known to play a major role in stabilization and structurization of the LPS layer in gram-negative bacteria (Nikaido and Vaara, 1985). Therefore, more rapid adsorption to the host cells in the absence of divalent cations might signify the alleviated sterical accessibility of the receptor on the cell surface due to less structured LPS layer.

It was also demonstrated that adsorption of bacteriophage Bam35 to the host cell is not dependent on divalent cations (II). In the case of bacteriophage ϕ13, the effect of EDTA was tested on the early stages of infection. It was determined that this chelator had no apparent effect on the entry stage following the adsorption, suggesting that bacteriophage ϕ13 binding to the receptor is neither dependent on the concentration of divalent cations in the medium (I).

1.3 Impact of the energetic state of the host on phage adsorption

The energetic state of the host cell sometimes affects the efficiency of phage binding. For example, bacteriophage PRD1 binding is dependent on the intracellular ATP concentration (Daugelavičius et al., 1997), while for adsorption of tailed bacteriophages T1 and ϕ80 energized CM of the host is required (Hancock and Braun, 1976). Such dependence can be dissected using energy poisons affecting different cellular processes (Nicholls and Ferguson, 2002).

Previous tests on PM2 adsorption revealed that it did not bind to the receptor if the host cells were not aerated (Kivelä et al., 2004). Based on this observation it was checked whether the presence of the receptor (in its active, phage binding-competent form) is dependent on the energetic state of the host cell (III). For this purpose a range of energy poisons specifically affecting cellular process linked to energy production and storing were used. The energy in bacterial cells can be stored in a form of intracellular ATP or as an electrochemical gradient across the CM also known as a proton motive force (PMF). The PMF consists of the membrane voltage ($\Delta\Psi$) and the pH gradient (ΔpH) across the CM (Mitchell, 1979). Notably, dissipation of the electrochemical gradient results in immediate cessation of ATP production and a consequent depletion of the intracellular ATP pool.

We performed a thorough analysis of the bacteriophage PM2 binding dependence on the energetic state of the host cells. For that, *Pseudoalteromonas* sp. ER72M2 cells were treated with a variety of poisons. First, the effect of compounds collapsing the PMF was tested. The treatment of ER72M2 cells with the proton ionophore dinitrophenol (DNP) efficiently reduced phage PM2 binding to the host cells (III, Fig. 1A). The same effect was observed when bacteria were treated with potassium cyanide (KCN). KCN inhibits cytochrome c oxidase, which leads to dissipation of the PMF and cessation of bacterial cell respiration.

Since the collapse of PMF had a drastic effect on PM2 adsorption, we set out to elucidate which component of the PMF is critical for the presence of active PM2 receptor on a host cell surface. For that, agents specifically affecting either $\Delta\Psi$ or ΔpH were used. Nigericin (NG) does not change $\Delta\Psi$ but affects the proton gradient by exchanging K^+ for H^+ across the biological membranes. It was observed that nigericin-treated cells adsorbed viruses normally (Fig. 1A). We then tested the effect of valinomycin (VAL) on PM2 binding. VAL transports K^+ down its electrochemical gradient by picking up K^+ on one side of the membrane, and releasing it on the other. Accordingly, in the presence of high extracellular K^+ concentration VAL dissipates the membrane voltage. Just like NG, VAL had no significant effect on PM2 adsorption (Fig. 1A).

As mentioned above, dissipation of the PMF has an immediate effect on the intracellular pool of ATP. To determine whether the presence of the receptor is dependent on the PMF or the ATP, effects of arsenate and azide were tested. Arsenate decreases intracellular ATP concentration via arsenolysis reaction. Azide reduces the ATP pool by inhibiting the cytochrome c oxidase and the membrane H^+-ATPase. Adsorption tests revealed that treatment of ER72M2 cells with azide and arsenate decreased PM2 binding by 40% and 24 %, respectively (Fig. 1A). These results are in good agreement with previous measurements showing that azide and arsenate decreased the intracellular ATP concentration of ER72M2 cells by ~55 % and ~26 %, respectively but had no prominent effect on $\Delta\Psi$ (Krupovič et al., 2007a). Moreover, incubation of ER72M2 cells with cyanide, not only dissipated the PMF, but also drastically decreased the

intracellular ATP concentration (Krupovič et al., 2007a). These data strongly suggest that inhibition of PM2 adsorption is associated with the drop in ATP level rather than collapse of the PMF.

Adsorption of cystovirus ϕ13 was also found to be dependent on the metabolic state of the host cell. Bacteriophage binding to the cells treated with azide or cyanide was significantly reduced. Both compounds blocked ϕ13 adsorption in a concentration-dependent manner (I, Fig. 3C). However, compared to PM2, adsorption inhibition effect was achieved at considerably higher concentrations of energy-depleting agents. Our results indicate that only actively respiring bacteria are suitable for ϕ13 infection.

In contrast to PM2 and ϕ13, bacteriophage Bam35 adsorption to the host cell was not affected by energy-depleting agents (II), suggesting that the receptor for Bam35 is a structure constantly present on the cell surface (see below). These results also suggest that different viruses have developed different strategies for host selection. Some viruses, like PM2 and ϕ13, seem to have adapted to infect only actively growing cells with high amounts of energy stored either in the form of ATP or PMF. Such cells are therefore likely to support successful virus progeny production. On the other hand, viruses like Bam35 seem to be less fastidious and infect even de-energized cells. However, the difference in the host selection tactics might be directly linked to the differences in the life cycles of these bacteriophages. It should be noted that PM2 and ϕ13 are both lytic phages, while Bam35 is a temperate virus capable of persisting silently within its lysogenized host. Therefore, even less metabolically active cells (given that they are not completely dead) seem to be a better choice for Bam35 when compared to the harsh extracellular environment.

1.4 The receptors

The receptor saturation assay in combination with the characteristics of phage binding to the host treated with energy-depleting agents helps to roughly estimate the abundance of the receptor and to understand its nature.

It was suggested that the receptor structure for bacteriophage ϕ13 is the LPS moiety which presence on the bacterial cell surface is irrespective of the energetic state of the host (Mindich et al., 1999). Surprisingly, treatment of the host with cyanide efficiently inhibited phage adsorption (I, Fig. 3C). Most probably the high cell surface concentration of H^+ ions due to cell respiration neutralizes the negative surface charge of the OM and results in better contact between the LPS and ϕ13 virions. However, a possibility remains that for irreversible binding ϕ13 uses a secondary receptor/co-receptor, presence of which is dependent on the active host metabolism. In addition, the relatively low number (~60) of the ϕ13 particles bound to the host cell (much more would be expected to bind if LPS is the sole receptor) also points to the possibility that in addition to the LPS there might be other factors crucial during the entry step following the binding to the LPS but preceding the fusion with the OM.

One *B. thuringiensis* HER1410 cell could bind hundreds of Bam35 particles (~500; II, Fig. 1C). This suggested that the receptor is very abundant structure on the cell surface. The outermost layer of gram-positive bacteria is the peptidoglycan. We purified peptidoglycan sacculi from the host cells (HER1410) and tested if they would interfere with phage adsorption. Incubation of Bam35 with HER1410-derived peptidoglycan efficiently reduced the number of infectious virions. The number of peptidoglycan-bound Bam35 virions was

dependent on the sacculi concentration (II, Fig. 2A).

We tested, if sacculi from other *Bacillus* strains are also able to bind and inactivate bacteriophage Bam35 particles. Peptidoglycan from four additional bacilli (alternative Bam35 host 4D22, resistant strain HER1410_R19, lysogenic strain HER1410_L5, and *B. thuringiensis* serovar wuhanensis 4T1) was purified and tested in the interference with phage adsorption assay. Cell walls from all the Bam35 infection-sensitive strains (i.e., not HER1410_R19) bound and inactivated bacteriophage Bam35 virions, although with different efficiencies (II, Fig. 2A). It is worth noting that results of phage inactivation with the cell walls derived from different *Bacillus* strains are in line with those of Bam35 adsorption to the intact cells. The resistant cell line HER1410_R19 did not bind Bam35 virions, neither did the sacculi isolated from these cells. The alternative host for Bam35, 4D22, bound virions less efficiently than HER1410, so did the 4D22-derived peptidoglycan.

To get further insight into Bam35 receptor, we removed teichoic acids (TA) from the peptidoglycan sacculi and tested Bam35 binding. Teichoic acid-less peptidoglycan of HER1410 bound phage particles with the same efficiency, while 4D22 and 4T1 sacculi without the TA adsorbed Bam35 even more efficiently when compared to the native ones. These results suggested that the cell wall TA are not necessary for bacteriophage Bam35 adsorption. Moreover, peptidoglycan from *E. coli* K-12 (gram-negative bacteria do not possess the TA) was also able to bind and inactivate Bam35 virions, further confirming that peptidoglycan itself, and not the TA, mediates the adsorption of Bam35 (II). In addition, we demonstrated that one of the two major sugar moieties constituting the peptidoglycan, *N*-acetyl-muramic acid (NAM), neutralized the virus, whereas the *N*-acetyl-glucosamine (NAG) did not (II, Fig. 2B). We have shown that the host cell peptidoglycan is necessary and sufficient for irreversible adsorption and inactivation of bacteriophage Bam35 and NAM moiety is involved in this process.

The high resolution structure of bacteriophage PM2 receptor-binding protein P1 revealed that it is structurally similar to a Ca^{2+}-dependent carbohydrate-binding protein from the marine bacterium *Saccharophagus degradans* (Abrescia et al., 2008). Accordingly, it was suggested that LPS might serve as a receptor for bacteriophage PM2 adsorption (Kivelä et al., 2008). However, our finding that PM2 adsorption is dependent on the intracellular ATP concentration argues against this possibility (III, Fig. 1). Since intracellular ATP cannot be used at the OM, where adsorption takes place, we suggest that PM2 receptor is most likely a protein molecule (or protein complex) spanning the cell envelope. As described above, the presence of the PM2-binding-competent form of the receptor is dependent on the intracellular ATP concentrations. Depletion of the intracellular ATP pool leads to either disassembly or conformational change in the PM2 receptor. We propose that PM2 receptor-binding protein P1 initially interacts with the sugar moiety of the LPS and this interaction facilitates the irreversible binding to the true receptor (III).

2. Phage penetration through the OM

Bacteriophages ϕ6, ϕ13 and PM2 infect gram-negative bacteria, which in addition to the cytoplasmic membrane and the peptidoglycan have an additional external layer, the outer membrane. The three phages do not possess the cell envelope puncturing devices resembling those of tailed dsDNA bacteriophages, nor do they form tube-like membrane structures as tectiviruses do. Consequently, they must employ different strategies to penetrate through the outer membrane. It is logical to assume that the viral membrane, a feature common to all three viruses, might play a role in this process. To investigate this possibility we employed measurements of outer membrane permeability to lipophilic compounds (I, III) and fluorescence de-quenching assay (I).

2.1 Measurements of OM permeability to lipophilic compounds

The LPS portion of the outer membrane of gram-negative bacteria forms a permeability barrier to lipophilic compounds, such as tetraphenylphosphonium (TPP^+) and ionophoroc antibiotic gramicidin D (GD). However, upon phage entry, the permeability of the OM to these compounds is expected to be (and indeed is) affected. As a result, this property can be conveniently employed to monitor the phage-induced effects on the OM during bacteriophage entry.

Bamford and co-workers (Bamford et al., 1987) have elegantly demonstrated that upon infection, the envelope of bacteriophage ϕ6 fuses with the OM of the host leading to liberation of the nucleocapsids into the periplasmic space. We followed the fluxes of TPP^+ across the envelope of infected and noninfected *P. syringae* cells by using ion selective electrodes. It was observed that both ϕ6 and ϕ13 induced an active uptake of TPP^+ by phage-sensitive *P. syringae* cells but had no effect on the resistant ones (I, Fig. 4A and B). The increased permeability of the OM of the infected cells to TPP^+ signifies fusion of the phage envelope with the bacterial OM; the phospholipids from the viral membrane create a permeability "window" to the lipophilic compounds in the LPS-composed outer leaflet of the OM. Notably, the accumulation of TPP^+ was only temporal lasting for ~3 and ~6 minutes in cells infected with ϕ13 and ϕ6, respectively. The transient nature of the permeability "window" could be explained by the lateral diffusion of the phage envelope-derived phospholipids in the plane of the OM. As a result, the permeability barrier of the OM is restored.

Corticovirus PM2, unlike cystoviruses, has an internal membrane covered with a protein capsid (Abrescia et al., 2008). However, it was demonstrated that phage binding to the receptor induces dissociation of the protein coat, composed of P1 and P2, exposing the phage lipid core to the host OM (Kivelä et al., 2004). Similarly to cystoviruses ϕ13 and ϕ6, the transient permeability of the host OM to lipophilic compounds, such as TPP^+ and the bulky ionophoric antibiotic GD, was observed during the first two minutes of PM2 infection, indicating that fusion between the phage and bacterial membranes takes place (Kivelä et al., 2004; Kivelä et al., 2008). It was proposed that PM2-derived membrane forms a temporal "window" for GD in the host OM. Notably, in the absence of calcium ions PM2-induced GD window remained in the host OM for several minutes (Kivelä et al., 2004). We extended this experiment and found that PM2-infected (but not noninfected) cells in the absence of

Ca^{2+} were sensitive to GD even twenty minutes after infection (III). These results suggest that phage-derived phospholipids stayed in the stable "patch" in the OM and that lateral diffusion of the phage lipids did not take place. It is possible that in the absence of Ca^{2+}, the dsDNA phage genome holds the phospholipid membrane patch in the OM via interactions with viral DNA-binding integral membrane proteins. We demonstrated that the fusion of PM2 membrane with the bacterial OM in the absence of calcium ions took place, but further steps of entry were abolished (III).

2.2 Labeling of phage membrane with R18

To monitor phage membrane fusion with host OM fluorescent lipid dye octadecyl rhodamine B (R18) was employed. We succeeded to incorporate the fluorescent probe into bacteriophage ϕ6 and ϕ13 envelopes to high enough concentrations to achieve significant self-quenching (I). During the fusion of viral envelope with the host cell OM the fluorescent probe was diluted leading to the decrease in self-quenching and concomitant increase in the fluorescence level. When R18-labeled bacteriophage particles were added to the host cell suspension, an increase in fluorescence started at about 10-15 s post infection. As expected, no considerable de-quenching of the fluorescent probe was observed when R18-labeled viruses were added to the resistant cells, indicating that increase in the fluorescence level in the case of phage-sensitive cells was infection-specific and associated with the fusion between the viral and cellular membranes.

Water molecules and small ions can freely move through bacteriophage capsids (Molineux, 2006). However, octadecyl rhodamine B, with molecular weight of 731.5 Da, was not able to penetrate through the capsid shells of inner membrane-containing corticovirus PM2 and tectivirus PRD1 (unpublished observation). Therefore, this technique could not be employed to study the entry of bacteriophage PM2.

3. Crossing the peptidoglycan layer

The peptidoglycan layer creates another barrier for an entering bacteriophage. Phages with larger genomes usually employ muralytic enzymes to create local openings in the murein sacculus to facilitate their genome delivery. The peptidoglycan degrading enzyme P5 of bacteriophage ϕ6 was described in substantial detail previously. It was demonstrated that P5 has an endopeptidase activity and is sensitive to elevated temperatures (Caldentey and Bamford, 1992). Based on virion protein composition analysis bacteriophage ϕ13 was predicted to have an equivalent virion-associated protein for peptidoglycan digestion (Qiao et al., 2000). We have demonstrated that ϕ13 virion indeed possesses a cell wall-degrading activity (I). Using spot test analysis and potentiometric measurements we determined that ϕ13 lytic enzyme is even more sensitive to elevated temperatures than that of ϕ6.

Bacteriophage PRD1, a type member of the *Tectiviridae* family, has two peptidoglycan-digesting enzymes associated with the virion, proteins P7 and P15. P7 was suggested to be the main muralytic enzyme involved in bacteriophage genome delivery, while P15 causes cell lysis at the end of the infection-cycle (Caldentey et al., 1994; Rydman and Bamford, 2002). Using zymogram analysis we have demonstrated that tetctivirus Bam35 also contains

muralytic activity associated with the virion (II, Fig. 4). As in the case of PRD1, two structural Bam35 proteins, gp26 and gp30, possess peptidoglycan-hydrolyzing activity. Interestingly, the muralytic enzymes of PRD1 do not share sequence similarity with those of Bam35 (Ravantti et al., 2003).

Previous zymogram analysis has also showed a muralytic activity associated with the bacteriophage PM2 virion. The PM2 protein responsible for peptidoglycan digestion was identified as the LC-associated integral membrane protein P7. Accordingly, P7 was concluded to play a role in genome delivery across the peptidoglycan layer of *Pseudoalteromonas* cells during PM2 entry (Kivelä et al., 2004). However, a doubt has been planted by Krupovič and co-authors as whether P7 does indeed possess a muralytic activity (Krupovič et al., 2007a). The argument was based on the fact that a positive signal in a zymogram assay is not a reliable indication of the peptidoglycan hydrolysis, as the same signal is generated when proteins tightly bind to the peptidoglycan. In addition, the authors found it unlikely that P7 would possess any enzymatic activity, as 22 out of 34 amino acids of P7 form the transmembrane domain (Krupovič et al., 2007a).

It was demonstrated that ER72M2 cells undergo abrupt lysis if the CM is permeabilized with cationic antibiotic Polymyxin B (PMB) or chloroform (Krupovič et al., 2007b). PM2 entry in the medium containing 2.5 mM Ca^{2+} and no Mg^{2+} ions leads to immediate lysis of the infected cells starting at ~3 minutes post infection (III, Figs. 3B and 4B). Notably, the physiological effects observed during the PM2 entry-related lysis were very similar to those recorded upon PMB-induced lysis of non-infected ER72M2 cells. Based on these findings we propose that entering bacteriophage PM2 relies on a cellular lytic factor for genome delivery across the peptidoglycan layer (III).

4. Bacteriophage penetration through the CM

Bacteriophage Bam35 virion architecture is very similar to that of PRD1. It was demonstrated that upon entry the inner membrane of PRD1 is transformed into a tube-like structure. Accordingly, it was proposed that this proteinaceous tubular membrane structure might form a channel for the genome delivery into the host cytoplasm (Bamford and Mindich, 1982; Lundstrom et al., 1979). In negatively stained Bam35 samples occasional empty "tailed" phage particles were observed, implying that Bam35, like PRD1, uses the tube-like membrane structure to cross the cell envelope and deliver its linear genome into the cell interior (Ackermann et al., 1978; Ravantti et al., 2003).

In contrast to the OM, the CM of bacterial cell is not permeable to small inorganic ions (such as K^+ and H^+) but allows the unrestricted movement of lipophilic compounds according to their electrochemical gradient (Nikaido and Vaara, 1985). Therefore, an efflux of intracellular K^+ is an indicator of the increased permeability of the cytoplasmic membrane, while distribution of the lipophilic cation TPP^+ across the CM can be monitored to follow the changes in membrane voltage in bacterial cells (Daugelavičius et al., 1997).

Since the gram-positive host cells of bacteriophage Bam35 do not possess the OM, the membrane voltage across the CM is the only factor determining the uptake of TPP^+ from the medium according to its electrochemical gradient. We have registered the changes in TPP^+ concentration in the medium during Bam35 infection. The release of the accumulated TPP^+ into the medium started about 1.5–2 minutes after Bam35 addition, signifying the depolarization of the CM (II, Fig. 5A). The depolarization of the CM indicates the

phage interaction with the CM, which is most likely associated with genome delivery into the host cytoplasm. Notably, Bam35-resistant, but capable of adsorbing the phage, HER1410_R20 cells showed no signs of membrane depolarization. This observation suggests that Bam35 binding to the peptidoglycan receptor had no effect on the CM and that in these cells an infection step following the phage adsorption, but preceding the genome penetration through the CM is compromised (II). It was also demonstrated that membrane depolarization was accompanied by a rapid leakage of intracellular K^+ starting 2 min post infection (Gaidelyte et al., 2005). In comparison, bacteriophage PRD1 entry also induced a temporal leakage of intracellular K^+ from the infected cells but did not depolarize the host CM (Daugelavičius et al., 1997). This suggests that even though the entry devices (membrane tube-like structures) of tectiviruses infecting gram-negative (PRD1) and gram-positive (Bam35) hosts are morphologically similar, the ways these structures are utilized for cell envelope penetration seem to be different.

We further investigated the effects of divalent cations on bacteriophage Bam35 genome delivery through the host CM. Our results indicated that addition of divalent cation-chelating agents EDTA or EGTA to the medium before infection abolished Bam35 entry into the host; no phage-induced effects on the CM were observed and the number of Bam35 infective centers was drastically decreased (II, Fig. 6A and Table 4). When added at excessive concentrations into the medium containing EGTA or EDTA, Ca^{2+} ions were able to rescue the Bam35 entry. Unexpectedly, no depolarization of the CM was observed (II, Fig. 6A). As mentioned above, the adsorption of Bam35 to the host cells is not dependent on divalent cations, suggesting that divalent cations play a role in the entry step following the adsorption (II).

To determine the divalent cation-dependent step in Bam35 entry, the chelating agents were added at different time points post infection. These experiments allowed us to determine that divalent cations are vital during 1.5-2 minutes of Bam35 infection, i.e., when phage interacts with the CM of the host. We also observed that depolarization of the CM during Bam35 entry was dependent on extracellular Ca^{2+} concentration. If the concentration of calcium ions in the medium was increased Bam35 entry into the host was unaffected but no depolarization of the CM was detected. This suggests that Bam35 genome entry and CM depolarization are coupled but separate processes.

It was demonstrated previously that, like in the case of bacteriophage Bam35, penetration of bacteriophage PM2 genome through the host CM caused depolarization of the membrane with accompanying leakage of the intracellular potassium into the medium (Kivelä et al., 2004). As mentioned above the natural habitat of phage PM2 is the sea water, containing ~10 mM Ca^{2+} and ~50 mM Mg^{2+}. This implies that divalent cations might play an important role during the PM2 infection-cycle. We tested this possibility by performing infection experiments at different divalent cation composition and/or concentration conditions (III). When magnesium ions were omitted from the growth medium no changes in PM2 propagation were observed, indicating that Mg^{2+} is not necessary for phage penetration into the cytoplasm or any other step of phage reproduction. However, variation of Ca^{2+} concentration in the absence of Mg^{2+} had a significant effect on the course of PM2 infection (III, Fig. 2B). We determined three threshold calcium ion concentrations (0.0 mM, 2.5 mM, and 5 mM) that had the most prominent effect on the growth of PM2-infected ER72M2 cells.

If the medium was devoid of calcium and magnesium ions PM2-infected cells neither grew nor lysed during the course of experiment (III, Fig. 2B). As mentioned above, binding of the phage to

the host in the absence of divalent cations was even more efficient than at the normal infection conditions (Fig. 2A). The fusion of the phage membrane with host OM was also found to be uncompromised. However, no depolarization of the CM was observed under such conditions (Kivelä et al., 2004). Accordingly, we suggested that the PM2 genome entry through the CM was affected. Interestingly, the arrested state of the PM2 infection could be relieved by addition of calcium ions later during infection. Adding 10 mM Ca^{2+} as late as 40 min post infection resulted in continuation of the PM2 replication cycle with concomitant release of progeny viruses (III, Fig. 5). Notably, the time from Ca^{2+} addition to the cell lysis was roughly the same as the duration of normal PM2 infection cycle. Tailed dsDNA bacteriophage 4Ic, also failed to penetrate into the host in the absence of calcium ions in the medium. However, addition of Ca^{2+} did not result in successful rescue of the phage entry (Landry and Zsigray, 1980). Notably, in the case of PM2, calcium ions could not be substituted by magnesium ions (III, Fig. 3A) pointing to a specificity of the PM2 entry process to calcium ions.

Interestingly, when ER72M2 cells were infected in the presence of 2.5 mM Ca^{2+}, sharp and complete clearance of the infected culture was observed. However, when the Ca^{2+} concentration was increased to 5 mM, the infected cells did not grow following phage addition, but lysed at the time of normal lysis (~50 min p.i.) and released comparable amount of phage progeny (III, Fig 2B). A recently developed potentiometric assay (Krupovič et al., 2007a) allowed us to determine the physiological response of ER72M2 cells to PM2 infection in the medium devoid of Mg^{2+} and containing 2.5 mM Ca^{2+}. Turbidity of the PM2-infected cell cultures as well as K^+ fluxes across the CM and dissolved O_2 concentration in the medium were monitored under these conditions (III, Fig. 4B). Up until 2 minutes post infection the infection process under low Ca^{2+} conditions (2.5 mM Ca^{2+}, no Mg^{2+}) closely resembled that in normal growth medium (10 mM Ca^{2+}, 50 mM Mg^{2+}). However, in contrast to the temporal and reversible genome-entry-associated potassium efflux recorded 2 min p.i. in normal SB medium, abrupt and complete leakage of intracellular K^+ was recorded under low Ca^{2+} conditions. The leakage of potassium coincided with a rapid decrease in cell culture turbidity and cessation of respiration (III, Fig. 4B), indicating the lysis of the infected cells (III, Fig. 3B).

Using PM2 entry-deficient mutant *sus2* (Kivelä et al., 2008) we provided evidence that PM2 binding to the host cell receptor did not cause lysis of the infected cells in low calcium containing medium (2.5 mM Ca^{2+}). Furthermore, we have employed PM2 lysis mutant M85 (Krupovič et al., 2006, Krupovič et al., 2007a) and demonstrated that lysis protein P18 is not involved in the PM2 entry-related cell lysis (Fig. 2C), suggesting that the early lysis and the progeny release-associated lysis are two independent phenomena governed by distinct factors.

However, under low Ca^{2+} conditions (2.5 mM Ca^{2+}, no Mg^{2+}) no progeny virus production was detected (III, Table 1). We then examined whether PM2 entry and replication inside the cell could be rescued if SB medium, in addition to 50 mM Mg^{2+}, was supplemented with 2.5 mM Ca^{2+}. Surprisingly, in these conditions the PM2 growth curve resembled the one obtained in divalent cation rich SB medium (Fig. 3). Moreover, the yield of PM2 progeny was of the same order of magnitude as during infection in normal SB medium (Table 1). We also determined that magnesium could be substituted by barium. In SB medium containing 2.5 mM Ca^{2+} and 50 mM Ba^{2+} PM2 entered the host without lysing it and virus production was also unaffected (Fig. 3, Table 1). These results strongly suggest that magnesium/barium are used to ensure the stability of the OM. Collectively our results indicate that at least 2.5 mM Ca^{2+} is needed for successful PM2 penetration through the CM. The OM of the

host has to be stabilized by divalent cations to avoid lysis of the PM2-infected cells early in the infection.

Bacteriophage ϕ6 nucleocapsid penetration through the host cytoplasmic membrane was studied in detail previously (Ojala et al., 1990; Olkkonen et al., 1990; Poranen et al., 1999; Romantschuk et al., 1988). To study the cystovirus entry-induced effects on the CM, the OM of the host was permeabilized with EDTA. EDTA removes divalent cations from the LPS layer resulting in increased OM permeability to lipophilic compounds. Measurements of TPP^+ fluxes across the CM indicated that ϕ6 and ϕ13 entry caused depolarization of the CM. Bacteriophage ϕ13 had a considerably stronger effect on membrane voltage than ϕ6 (Fig. 4C and D). These results are in good agreement with the number of particles entering the cell. If an MOI of 10 is used in the case of ϕ6 only one or two particles enter the host, while ~10 ϕ13 virions could enter the host under the same MOI. In addition we showed that during cystovirus entry the CM depolarization was accompanied by the efflux of intracellular potassium, which started to leak at ~3 minutes post infection and ceased at about 10 minutes post infection (I, Fig. 5C).

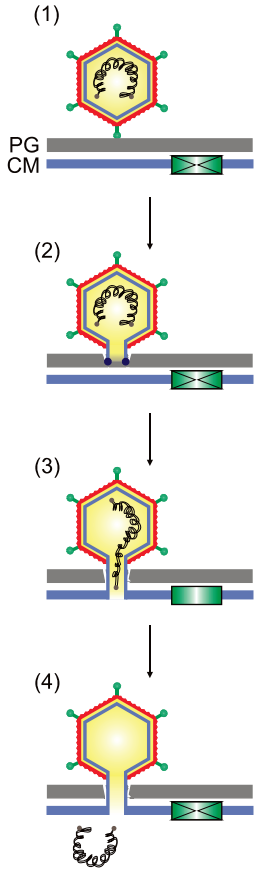
E. CONCLUSIONS AND FUTURE PERSPECTIVES

This study concentrated on the first step of bacteriophage infection: entry into the host cell. To reach the site of replication, the host cell cytoplasm, phages have to overcome the multilayered bacterial cell envelope.

We studied the entry of membrane-containing bacteriophages ϕ6, ϕ13 (*Cystoviridae*), Bam35 (*Tectiviridae*) and PM2 (*Corticoviridae*) into their host cells. The investigation of the discrete steps of phage penetration, (adsorption to the cell surface, penetration through the OM, crossing the peptidoglycan layer, and penetration through the CM), in combination with previously obtained results enabled us to propose plausible mechanistic models for the entry of these phages into their hosts.

Our results indicate that membrane-containing bacteriophages belonging to different virus families use completely different strategies to enter into their hosts. This suggests that virion architecture does not dictate the entry mechanism.

Bacteriophage Bam35 entry into the host cell



PG – peptidoglycan layer;
CM – cytoplasmic membrane

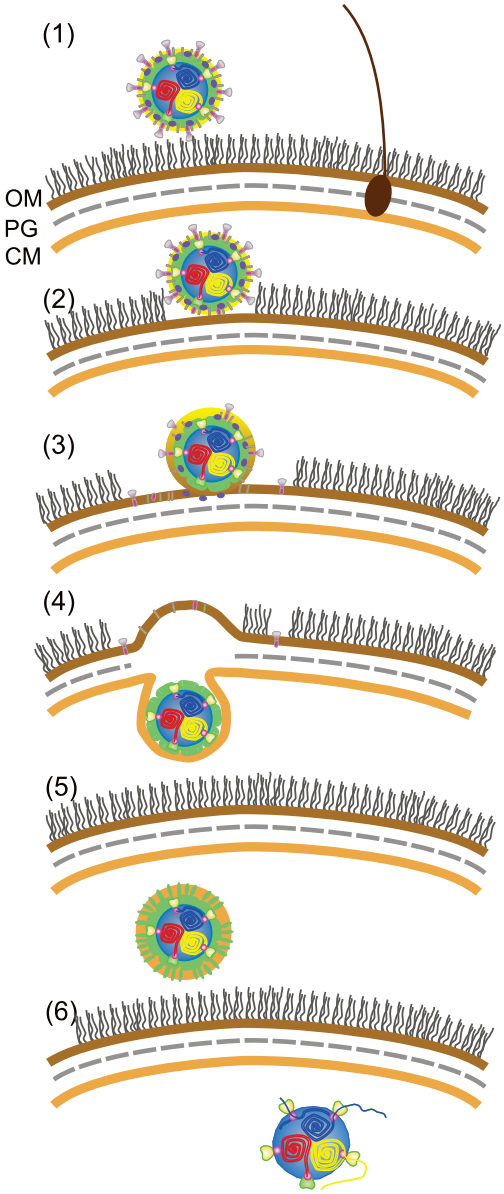
(1) Bacteriophage Bam35 rapidly adsorbs to the peptidoglycan layer of the host; the *N*-acetyl-muramic acid moiety serves as a receptor for the phage. Binding to the receptor is independent of the energetic state of the host cell.

(2) The spike complex dissociates from the vertex creating an opening in the capsid. Phage membrane is transformed from spherical into the tube-like structure. Virion-associated muralytic enzyme(s) locally degrades the peptidoglycan layer.

(3) The tube-like membrane structure penetrates through the peptidoglycan and fuses with the CM. Entering bacteriophages induce a temporal opening of an ion permeable channel in the host CM. This step of entry requires Ca^{2+} ions.

(4) Bam35 dsDNA genome enters the host cytoplasm, leaving the empty capsid associated with the host cell surface.

Bacteriophage ϕ13 entry into the host cell



OM – outer membrane;
PG – peptidoglycan layer;
CM – cytoplasmic membrane.

(1) Bacteriophage ϕ6 binds to the type IV pilus (Bamford et al., 1976), while ϕ13 adsorbs directly to the LPS (Mindich et al., 1999). However, the possibility remains that ϕ13 uses a secondary receptor/co-receptor, the presence of which is dependent on the active metabolism of the host (for irreversible binding). All the following steps are shown for phage ϕ13.

(2) Interaction with the receptor leads to structural rearrangements in the virion structure. Consequently, the spike complex is removed.

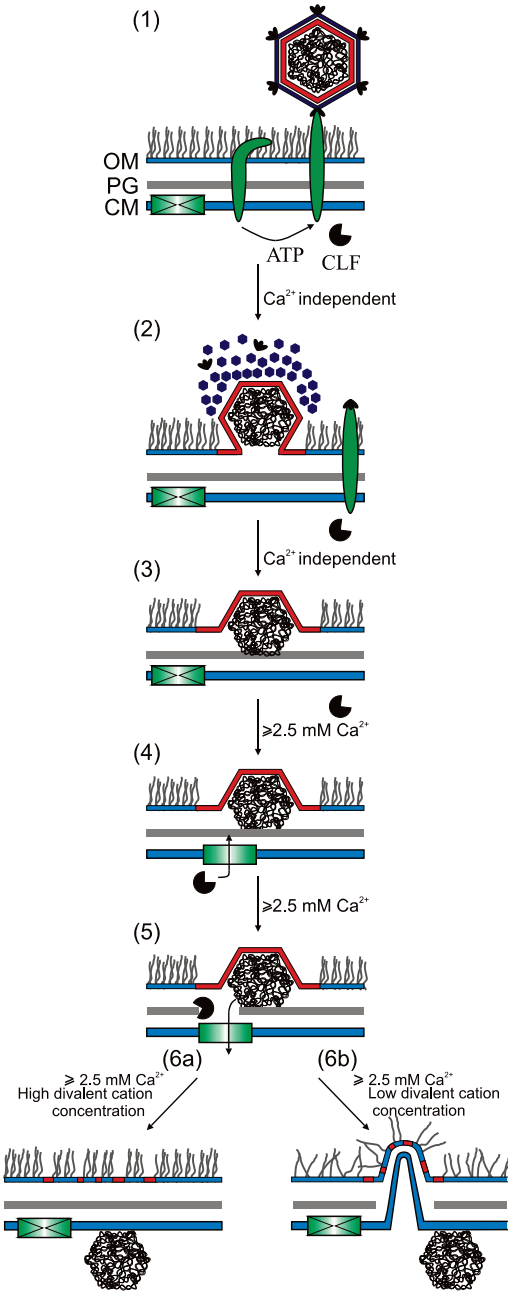
(3) Phage protein-mediated fusion between the ϕ13 envelope and the host OM takes place. After the fusion, the nucleocapsid is released into the periplasmic space.

(4) Nucleocapsid-associated muralytic enzyme makes a local opening in the peptidoglycan layer and allows the nucleocapsid to interact with the CM.

(5) Invagination of the CM leads to penetration of the nucleocapsid into the cell interior via the endocytosis-like event (Romantschuk et al., 1988).

(6) The nucleocapsid is liberated from the intracellular "endocytotic" membrane vesicle, the outer protein shell dissociates from the nucleocapsid, the polymerase complex is activated and genome replication takes place.

Bacteriophage PM2 entry into the host cell



OM - outer membrane; PG - peptidoglycan layer; CM - cytoplasmic membrane; CLF - cellular lytic factor. Green box depicts the pore in the CM.

(1) PM2 binds to the receptor, the presence of which on the host cell surface is dependent on the intracellular ATP concentration.

(2) Receptor binding triggers the dissociation of the capsid shell composed of proteins P1 and P2 (Kivelä et al., 2004). PM2 lipid core is exposed to the OM. Phage protein P10 mediates fusion between the viral membrane and the OM (Kivelä et al., 2008).

(3) Phage genome enters the periplasm and faces the peptidoglycan layer.

(4) This step of PM2 entry is strictly dependent on calcium ions. In the absence of Ca^{2+}, PM2 infection is arrested. Phage-derived phospholipid patch stays in the OM and entry of the genome is blocked.

PM2 induces opening of a pore in the CM, which allows intracellular CLF to enter the periplasmic space.

(5) The CLF locally degrades the cell wall and PM2 genome penetrates into the cytoplasm through the same pore. This step of PM2 entry requires at least 2.5 mM concentration of calcium ions.

(6a) After phage genome entry the pore in the CM closes, peptidoglycan layer is repaired and virus replication begins.

(6b) However, in the absence of divalent cations the PM2 DNA enters the cytoplasm, the pore in the CM closes, but the unsealed opening in the cell wall and reduced rigidity of the unstructured LPS layer lead to the osmotic lysis of the infected cell.

The entry mechanism utilized by bacteriophages ϕ6 and ϕ13 is unique among bacterial viruses and resembles that employed by animal viruses. Notably, endocytosis in prokaryotes has not been previously reported. Therefore, it would be of great interest to (i) identify the host proteins involved in the phage entry-mediated formation of the CM invagination, (ii) elucidate how the polymerase core is released from the endocytotic vesicle into the host cytoplasm.

Bacteriophage Bam35 is structurally very similar to the well-described type member of the *Tectiviridae* family, phage PRD1. Bam35 and PRD1 infect gram-positive and gram-negative bacteria, respectively. The two viruses use different receptors implying that their receptor-binding spikes might also be different. The spike complex of PRD1 has been studied in great detail, while the proteins comprising the spike structure in Bam35 are yet to be identified. A number of PRD1 membrane proteins were shown to be involved in phage genome delivery, whereas information on the role of Bam35 proteins in phage entry is still obscure. Identification and functional characterization of these proteins would therefore be of great interest.

In this study the mechanism for bacteriophage PM2 entry into the host cell was proposed. However, there are still questions that need to be addressed. For example, data presented here imply that the receptor for phage PM2 is a protein molecule. However, the nature of this protein is still enigmatic. Similarly, despite apparent involvement of a cellular lytic factor in the PM2 entry into and egress from the host, the exact host protein is still not known. PM2 genome is highly supercoiled and it would be of a great interest to elucidate how the viral DNA penetrates through the CM and in what topological form it is delivered into the host cytoplasm: as a supercoiled circular, relaxed circular or linear DNA molecule, double- or single-stranded.

The work presented here provides the basis for further research on the entry of membrane-containing bacteriophages into their host cells.

F. ACKNOWLEDGEMENTS

This work was carried out in the Finnish Academy Centre of Excellence in Structural Virology / Virus Research (2000-2005 and 2006-2011), Institute of Biotechnology and Department of Biosciences, at the University of Helsinki, under the supervision of Professor Dennis Bamford.

I am grateful to Dennis for taking me into his lab, for generously sharing his knowledge and experience. Your enthusiasm and wisdom have guided my work through this thesis project which was not always going as smoothly as I would have liked it to. I am also grateful for your endless patience.

I want to thank Professors Mikael Skurnik and Martin Romantschuk for careful revision of my thesis, expert advice, and for providing valuable suggestions that helped improving the thesis manuscript. Warm thanks to Docent Kaarina Lähteenmäki and Professor Mikael Skurnik for their support and advice on my work as members of my Follow-up group.

The Viikki Graduate School in Molecular Biosciences (VGSB) is thanked for the support provided in the course of this thesis work. I am grateful for the courses organized by the school and for the financial support that made it possible for me to finish my PhD thesis project and attend conferences.

I am deeply grateful to all the co-authors, especially Rimantas Daugelavičius and Aušra Domanska, for their valuable and rewarding contributions to this thesis work.

My special thanks are reserved for the members of the Membrane biochemistry lab in Vilnius University where I got my introduction to research work years ago. I am very grateful for all the moments we shared together.

I owe my most sincere gratitude to my former supervisor Rimantas for his enthusiastic and encouraging attitude, professional approach and support. I am grateful for all the lively scientific and not-so-scientific discussions we had.

I am also in great debt to the skilled technical personnel of the Bamford lab for providing such a smoothly working infrastructure.

Elina, Aušra, Silja, Petra, Andrius, Miao, Ana, Mart, it was a pleasure to share the office with you.

I would like to thank my colleagues and friends Greta, Andrius, Simonas and his beautiful family, Rimantas, Eglė, and Ana for their friendship, much valued company and all the nice moments we shared.

I am especially pleased to get to know Vio, who became my friend. Thank you for the lengthy and refreshing coffee breaks and for sharing your ups-and-downs and gossips with me.

I am very pleased that the International Committee on Taxonomy of Viruses (ICTV) has recognized my contribution to the field of Virology and named a virus family, the *Virgaviridae*, after me.

My heartfelt thanks go to my parents for their love, support, and for having faith in my abilities. My mom and my mother in law are warmly thanked for taking care of our son, while I and Mart were attending conferences.

Finally, I want to thank my family. My extraordinary husband Mart, you have supported me in every possible and impossible way. Thank you for substantial improvement of my thesis by making constructive and critical comments and changes to the manuscript. Our wonderful son Matas, you make me so proud.

Virga
Helsinki, May 2010

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