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Phospholipids of lipid-containing bacteriophages and their transbilayer distribution

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ACADEMIC DISSERTATION

To be discussed publicly, with permission of the Faculty of Biosciences of the University of Helsinki, in the auditorium 2041 of Biocenter 2, Viikinkaari 5, Helsinki, on March 18th, 2008, at 12 noon.

Helsinki 2008

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© Simonas Laurinavičius 2008
ISBN 978-952-10-4598-1 (paperback)
ISBN 978-952-10-4599-8 (PDF, http://ethesis.helsinki.fi)
ISSN 1795-7079

Yliopistopaino, Helsinki University Printing House
Helsinki 2008

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ACKNOWLEDGEMENTS

This work was carried out at the Centre of Excellence in Structural Virology/Virus Research (2000-2005 and 2006-2011) of the Academy of Finland, at the Institute of Biotechnology and Department of Biological and Environmental Sciences, and at the Institute of Biomedicine, Department of Medical Biochemistry and Developmental Biology, at the University of Helsinki during 2003-2007. It was supported by Academy of Finland, Sigrid Juselius Foundation, Kazys Martinkus Memorial Foundation and Helsinki Biomedical Graduate School.

My deepest gratidute (multiplied by 2) is shared between my two supervisors, Professor Dennis Bamford and Docent Pentti Somerharju. I am indebted for their patience, when I was learning things in their labs, as well as for the encouragement, extra motivation and "multifunctional" support, when something was not going the way it should. I could turn to Dennis or Pentti with any problem, be it professional or personal (and I was never hesitating to do that), and they were always giving me useful advices.

Professor J. Peter Slotte and Docent Vesa Olkkonen are thanked for a quick but careful reviewing of the thesis manuscript and for their useful comments. Docents Tero Ahola and Tuomas Haltia are thanked for their advices during the follow-up process of my thesis.

Docent Reijo Käkelä, who introduced me to the mass-spectrometry of lipids and to the fatty acid analysis, is warmly thanked not only as a co-author of my thesis papers, but also for *orienteering* me through my first years in Finland and in Somerharju lab. Your friendship has helped me in many ways. I also thank Dr. Anne Käkelä who also helped me with the fatty acid analysis in a finely small lab in Lahti.

I warmly thank all the past and present members of the Bamford and Somerharju laboratories. All of them were very helpful and friendly to me during my stay. You created the atmosphere in which working or just being was a pleasure. Marja-Leena Perälä, Sari Korhonen, Petri Papponen and Tarja Grundström are thanked also for skillful technical assistance. Due to the *lack* of the „language barrier“, I was mostly socializing with the Lithuanian members of the Bamford group (Mart, Virginija, Rimantas, Gabija, Aušra, Greta), and I thank them for sharing their time, thoughts and coffee with me.

I thank Docent Päivi Ojala and all members of her group for a warm welcome to their lab and, especially, for the support during the final stages of my thesis writing.

I also thank my Lithuanian friends outside the scientific community for the fine moments when playing basketball, watching Formula-1, discussing political issues or just drinking "that drink from the cups".

I wish to dedicate this thesis to all my relatives. Most of all, I thank my parents, Nijolė Laurinavičienė and Eligijus Laurinavičius, for their love and support, especially during the most difficult moments. My brother Jurgis (and his family) and sister Karolina are thanked for the support and the fun moments during the short holidays in Lithuania. The support of my parents-in-law is also appreciated.

All the warmest words and feelings go to my wife Rūta Saulytė-Laurinavičienė for her love and support during our time together, and for accepting my love as well. Kai kurie dalykai atrodo daug paprastesni, kai šalia esi Tu ir Joriukas.

Simonas Laurinavičius
Helsinki, March 2008

ORIGINAL PUBLICATIONS

This thesis is mainly based on the following articles that are referred to in the text by their Roman numerals.

I. Laurinavičius, S., Käkelä, R., Somerharju, P., and Bamford, D. H. (2004) **Phospholipid molecular species profiles of tectiviruses infecting Gram-negative and Gram-positive hosts**, *Virology 322*, 328-336.

II. Laurinavičius, S., Käkelä, R., Bamford, D. H., and Somerharju, P. (2004) **The origin of phospholipids of the enveloped bacteriophage phi6**, *Virology 326*, 182-190.

III. Laurinavičius, S., Bamford, D. H., and Somerharju, P. (2007) **Transbilayer distribution of phospholipids in bacteriophage membranes**. Biochim Biophys Acta 1768: 2568-2577

In addition, some unpublished results are presented.

ABBREVIATIONS

CL – cardiolipin

CM – cytoplasmic membrane

ESI-MS – electro-spray ionization mass-spectrometry

FA – fatty acid (FAs = fatty acids)

OM – outer membrane

PA – phosphatidic acid

PC – phosphatidylcholine

PE – phosphatidylethanolamine

PG – phosphatidylglycerol

PI – phosphatidylinositol

PL – phospholipid (PLs = phospholipids)

PM – plasma membrane

PS – phosphatidylserine

SM – sphingomyelin

TNBS – trinitrobenzene sulfonic acid

ABSTRACT

In this study we used electro-spray ionization mass-spectrometry to determine phospholipid class and molecular species compositions in bacteriophages PM2, PRD1, Bam35 and phi6 as well as their hosts. To obtain compositional data of the individual leaflets, phospholipid transbilayer distribution in the viral membranes was studied. We found that 1) the membranes of all studied bacteriophages are enriched in phosphatidylglycerol as compared to the host membranes, 2) molecular species compositions in the phage and host membranes are similar, and 3) phospholipids in the viral membranes are distributed asymmetrically with phosphatidylglycerol enriched in the outer leaflet and phosphatidylethanolamine in the inner one (except Bam35). Alternative models for selective incorporation of phospholipids to phages and for the origins of the asymmetric phospholipid transbilayer distribution are discussed.

Notably, the present data are also useful when constructing high resolution structural models of bacteriophages, since diffraction methods cannot provide a detailed structure of the membrane due to high motility of the lipids and lack of symmetric organization of membrane proteins.

REVIEW OF THE LITERATURE

1.1 Introduction

Viruses are obligatory parasites that are able to replicate only within an appropriate host cell by taking over the host's cellular machinery. Viruses are the most abundant biological entities on Earth as their number is at least ten times larger than the number of their hosts (Bergh et al. 1989; Wommack and Colwell 2000; Hendrix 2002). Organisms from all domains of life (*Eukarya*, *Archaea* and *Bacteria*) are infected by viruses. A major part of research in virology is aimed at combating the viral diseases of humans, domesticated animals, and plants in order to reduce their suffering and minimize the economical losses caused by viruses.

Viruses are molecular assemblies of protein, nucleic acid and, often, lipids as well. The replication of viral components and assembly of new virions is a consequence of many reactions that are relevant in other biological systems as well. Therefore, viruses are suitable model systems to study various aspects of molecular biology. Bacterial viruses (i.e. bacteriophages or phages) are very valuable model systems when studying the assembly of macromolecular structures as well as molecular mechanisms for genome replication and packaging, due to their relatively simple composition, easy propagation and the possibility to genetically manipulate them and their hosts. Phages are also used in practical applications, such as gene therapy, phage therapy, phage display (Clark and March 2006) and self-assembly of nano-surfaces onto which metal ions can be deposited to form nanowires that may be used in lithium-ion batteries (reviewed by Gazit 2007).

Lipid-containing bacteriophages contain a lipid membrane which is derived form the cytoplasmic membrane of the host and is involved in the fusion with bacterial membranes to aid viral entry and/or genome delivery. Lipid-containing bacteriophages have been particularly useful when studying factors influencing the structure, assembly and composition of viral membranes in general. Previous studies have addressed the phospholipid (PL) class and fatty acid (FA) compositions of some bacteriophages, but neither the detailed molecular species compositions nor the PL compositions of individual leaflets have been determined. Such data would be useful to interpret high-resolution structural models of bacteriophages, since diffraction methods cannot provide a detailed structure of the membrane due to high motility of the lipids and lack of symmetric organization of membrane proteins. Development of sensitive mass-spectrometric methods with high resolving power now allows one for the first time to determine phospholipid compositions of phage membranes and individual leaflets in great detail.

1.2 Bacterial membranes

1.2.1 Cell envelope of Gram-negative bacteria

Bacteriophages that infect Gram-negative bacteria have a more difficult task than those infecting Gram-positive hosts, since they encounter a triple-layered cell wall (**Figure 1**).

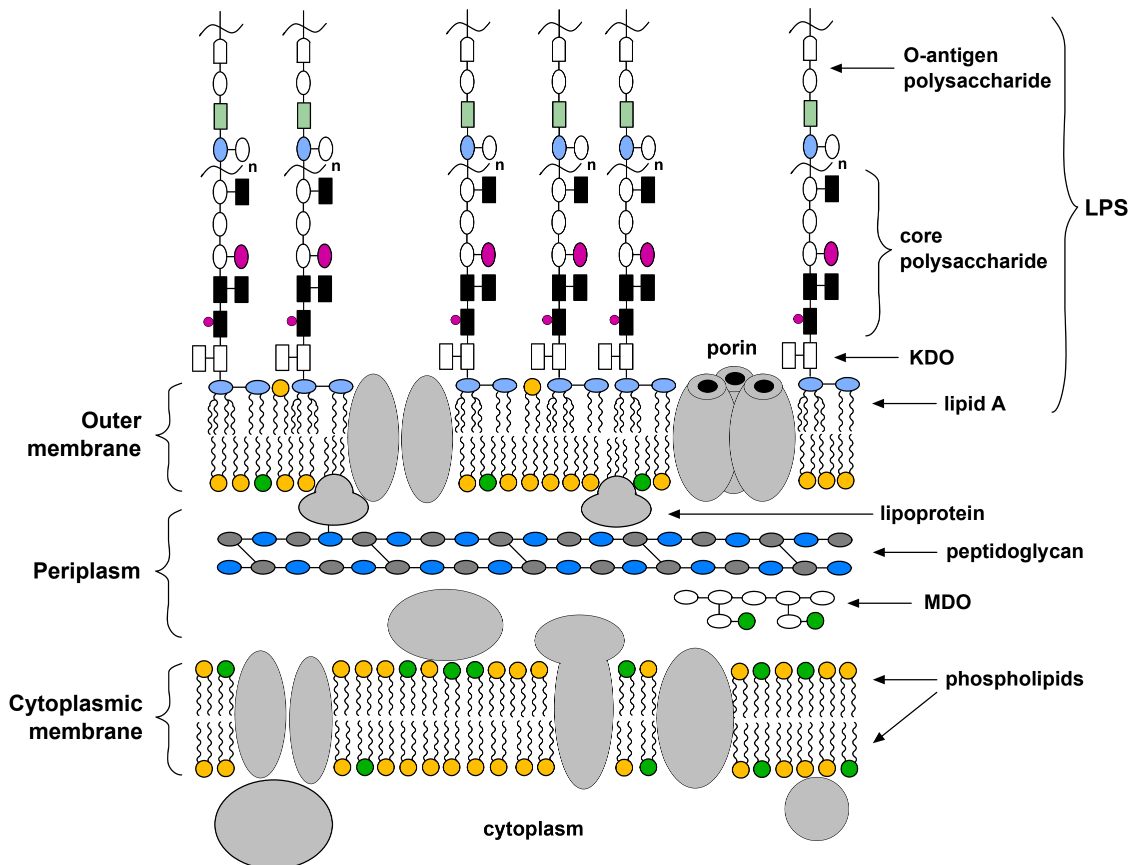

Figure 1. Schematic representation of the envelope of gram-negative bacteria *Escherichia coli*. Abbreviations: LPS – lipopolysaccharide, KDO – keto deoxyoctonate, MDO – membrane-derived oligosaccharides. Phospholipids with yellow headgroups represent PE, and those with green ones – PG. CL is not shown. Phosphoglycerol moiety from PG is also attached to MDO. The picture is redrawn from Raetz and Whitfield (2002).

The three cell wall layers of Gram-negative bacteria are: 1) the cytoplasmic membrane which surrounds the cellular cytoplasm; 2) the peptidoglycan layer which defines the shape of bacterium and protects it from osmotic stress, and 3) the outer membrane which is the outermost layer of the cell wall and functions as a permeability barrier for harmful compounds and yet allows necessary solutes to reach the CM. The structure of Gram-positive cell wall differs from that of Gram-negative bacteria as it contains only the CM and a thick peptidoglycan layer to (Matias and Beveridge 2005) which teichoic and lipoteichoic acids are attached.

The OM of Gram-negative bacteria is quite a heterogeneous structure. The outer surface of this membrane is almost entirely covered by lipopolysaccharide, while the inner leaflet is composed of phospholipids. There are three main components in the lipopolysaccharide – a hydrophobic lipid A region, which almost entirely constitutes the outer layer of the OM bilayer, the core, and the hydrophilic O-antigen polysaccharide region interacting with the aqueous medium (Osborn et al. 1964). The protein content of the OM is quite high, but it contains only a limited number of protein species: the minor enzymes phospholipase A_1 and protease, several protein species which are involved in specific diffusion processes as well as two major protein species, murein lipoprotein and porins (Osborn and Wu 1980). Part of the murein lipoprotein molecules are covalently linked to the peptidoglycan (Braun and Wolff 1970). Membrane spanning porins are trimeric and form pores or channels for the free non-specific flow of small hydrophilic solutes across the OM (Hancock 1987; Nikaido 2003). Proteins of the bacterial OM (Heller 1992) as well as components of the lipopolysaccharide (Daugelavičius et al. 2005) can serve as receptors for bacteriophages.

Below the outer membrane resides the peptidoglycan (murein) layer. It is composed of the glycan backbone of N-acetylglucosamine and N-acetylmuramic acid linked together into long oligosaccharide chains. These chains are interlinked together via short oligopeptides. The thickness of the peptidoglycan layer ranges from 2.5 to ~7 nm in Gram-negative bacteria (Labischinski et al. 1991; Yao et al. 1999). The space between the OM and CM where peptidoglycan is located is called the periplasmic space, which also contains some proteins and membrane-derived oligosaccharides (MDO, Kennedy 1982).

The CM of Gram-negative bacteria consists of phospholipids, which form a bilayer, and many membrane proteins that are inserted in it or peripherally associated with it. Two thirds of the cellular phospholipids are located in the CM (the rest being in the inner monolayer of the OM). Most of the enzymes required for oxidative phosphorylation, translocation of OM constituents, uptake of sugars and amino acids and cell division (at least 100 major protein species) are found in the CM (Kadner 1996). Also almost all enzymes required for PL biosynthesis are associated with the CM. Bacterial PLs are synthesized in the inner leaflet of the CM (Shibuya 1992) and then rapidly translocated to the outer leaflet, possibly by unspecific lipid transporter (Kol et al. 2002). In Gram-negative bacteria, phospholipids from the CM are rapidly transported to the OM (Jones and Osborn 1977; Donohue-Rolfe and Schaechter 1980).

1.2.2 Phospholipid synthesis

The major PL classes in Gram-negative bacteria are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). Small amounts of phosphatidic acid (PA), phosphatidylserine (PS), lysophospholipids and diacylglycerol, which are intermediates of biosynthetic pathways or turnover products, may also be present.

Fatty acids of the phospholipids are synthesized in the cytosol by several rounds of repeating reactions. First, the precursor acyl-ACP [an acyl group (e.g., acetyl group to start with) bound to acyl carrier protein] is condensed with malonyl-ACP by β-ketoacetyl-ACP synthetase (type I). The resulting β-ketoester is then reduced by NADPH-dependent reductase, and the nascent β-D-hydroxyacyl-ACP is dehydrated by a dehydrase. The produced trans-2-unsaturated acyl-ACP is reduced to generate a saturated acyl-ACP which is two carbon atoms longer than the original acyl chain at the start of the cycle. Unsaturated acyl-ACP can be produced by the isomerase activity of β-hydroxydecanoyl-ACP dehydrase. This enzyme catalyzes conversion of *trans*-decanoyl-ACP to *cis*-decenoyl-ACP which, after subsequent reduction and several new rounds of elongation, is converted to the unsaturated FA of the required length (Cronan and Rock 1996; Campbell and Cronan 2001).

The pathways for the biosynthesis of the bacterial PLs are depicted in **Figure 2.**

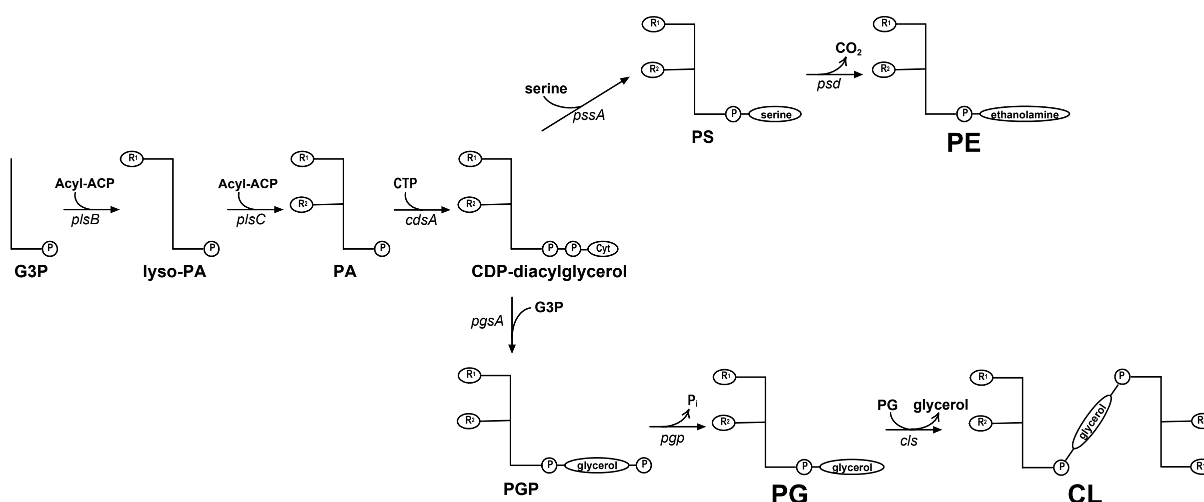

Figure 2. Phospholipid biosynthesis in Gram-negative bacteria. The genes encoding for the enzymes involved in the biosynthesis are indicated (in *Italics*) close to the arrows: *plsB* – G3P acyltransferase, *plsC* – 1-acyl-G3P acyltransferase, *cdsA* - CDP-diacylglycerol synthetase, *pssA* – PS synthetase, *psd* – PS decarboxylase, *pgsA* – PGP synthetase, *pgp* – PGP phosphatase, *cls* – cardiolipin synthetase.

Phospholipid synthesis takes place in the CM, since most of the enzymes catalyzing various steps of PL synthesis are membrane-bound and their catalytic domains reside in the cytoplasm (Raetz 1978). The only reaction of the phospholipid biosynthetic pathway (not shown in **Figure 2**) which takes place in the cytosol is the conversion of dihydroxyacetonephosphate to glycerol-3-phosphate (G3P) by the action of G3P dehydrogenase. This step is essential since strains that are defective in this enzyme require G3P in the growth medium (Bell 1974).

The membrane-associated synthesis of PLs starts by the addition of one fatty acid (from acyl-ACP) to G3P yielding lyso-PA, followed by the addition of another FA to produce PA. Usually, a saturated FA is attached at the 1-position of the glycerol backbone, while an unsaturated FA is attached to the 2-position (Cronan and Rock 1996). Only trace amounts of

PA are found in Gram-negative bacteria because this lipid is rapidly condensed with CTP to generate CDP-diacylglycerol, which is the last common precursor in the biosynthesis of all membrane PLs. From here on CDP-diacylglycerol can be metabolized via two different pathways. First, it can be condensed with serine to produce PS by PS synthetase, which becomes activated only when associated with the CM (Saha et al. 1996). PS is subsequently decarboxylated to generate PE, the most abundant PL in Gram-negative bacteria. Alternatively, another molecule of G3P can react with CDP-diacylglycerol to produce phosphatidylglycerolphosphate (PGP), which is then dephosphorylated yielding PG, the second major lipid of Gram-negative bacteria. Unlike in eukaryotes, in which CL is synthesized from one molecule of CDP-PG and one molecule of PG, in bacteria CL is synthesized from two PG molecules (Cronan and Rock 1996).

1.2.3 Regulation of phospholipid composition in bacteria

PE, PG and CL comprise ~75%, ~20% and ~5% of total PLs of Gram-negative bacteria, respectively. The three most abundant FAs are palmitic (16:0), palmitoleic (c9-16:1) and *cis*-vaccenic (c11-18:1) acids.

The FA composition depends on the growth temperature since the fluidity of bacterial membranes is regulated by the proportion of saturated versus unsaturated FAs. For instance, if the growth temperature is lower than the normal growth temperature, the proportion of *cis*-vaccenic acid in newly synthesized phospholipids increases at the expense of palmitic acid (Marr and Ingraham 1962). At lower temperature, the enzyme β-ketoacetyl-ACP synthetase II catalyzes elongation of palmitoleic acid faster than the type I enzyme (Magnuson et al. 1993), thus increasing the proportion of *cis*-vaccenic acid among the newly synthesized FAs already after 30 sec upon down-shift of the temperature (Garwin and Cronan 1980). The PL class composition (headgroup composition) remains constant with temperature.

The phospholipids in bacterial membranes form a bilayer, i.e., a lamellar phase. In contrast, the lipids extracted from *E.coli* form a non-lamellar phase under conditions that are close to physiological ones. This is probably due to a high amount of PE in bacterial membranes, since unsaturated PE can form the inverted hexagonal phase (de Kruijff 1997). It has been shown that phospholipids in *E.coli* membranes are very close to forming non-lamellar phases, and by regulating the fatty acid chain length the bacteria are able to maintain their membranes close to the transition from bilayer to hexagonal phase. This may be necessary for proper insertion and function of membrane proteins (van Dalen and de Kruijff 2004).

Evidence for polymorphic regulation of PL headgroup composition came from genetic studies of bacterial phospholipid biosynthesis. Mutants defective in PS synthetase almost completely lack PE and can survive only in the presence of divalent cations (Ca^{2+}, Mg^{2+}, Sr^{2+}, but not Ba^{2+}). Since these mutants contain elevated levels of CL, it was suggested that upon binding divalent cations CL assumes the shape of a cone and thus mimics PE. The

bacteria cannot survive if both PE and CL are lacking. It has been suggested that CL plays a role in the survival of bacteria since CL levels are elevated during stationary growth phase due to increased CL synthetase (Hiraoka et al. 1993).

When PS synthetase is overexpressed, no elevation of PE levels is observed (Ohta et al. 1981a). Similarly, overexpression of PGP synthetase does not lead to increased levels of PG or CL (Ohta et al. 1981b). Even when the synthesis of PG was substantially increased by treating wild-type *E.coli* cells with arbutin [a beta-glucoside acceptor of the phosphoglycerol moiety from PG (Bohin and Kennedy 1984)], neither the level of PGP synthetase nor synthesis of PE were affected (Jackson et al. 1986). These observations lead to the proposal that PL biosynthesis in E.coli is quite tightly regulated and that the level of acidic PLs in the membrane plays a regulatory role (Saha et al. 1996). It has been suggested that the cytosolic ribosome-associated PS synthetase enzyme binds to the membrane by sensing slight increases of PG/CL levels therein and is thus activated to restore normal levels of PE.

1.3 Biogenesis of viral membranes

Viruses do not have the capacity to encode all the enzymes needed for the synthesis of viral components. As parasites they take advantage of the cellular enzymatic machinery (e.g., ribosomes) or use the components already available in the cell (nucleotides, lipids). Accordingly, viral membranes usually contain proteins encoded by the viral genome and phospholipids which originate from the host. The constituents of viral membranes and the forces that drive the formation of viral membranes often differ among various types of viruses. Four distinct mechanisms of the viral membrane (or other membrane of defined size, shape and lipid composition) assembly are depicted in **Figure 3**.

According to the first mechanism (**Figure 3**, model 1), binding of a viral core to the membrane provides the driving force for the membrane deformation. This initial deformation is followed by adherence of additional membrane to the viral core until the enveloped core is released on the opposite side of the parent membrane. This is the mechanism by which most of the enveloped animal viruses obtain their membrane (Cadd et al. 1997; Garoff et al. 1998). In order to initiate this process, the viral core should be pre-assembled and the proteins for the recognition of the core should be inserted into the host membrane. By this mechanism, the core determines the size and shape of the membrane, but the lipid composition would depend on the specificity of the inserted proteins. The topology of the viral membrane remains the same as that of the parent membrane. Similarity in the PL transbilayer distribution in vesicular stomatitis virus, Semliki Forest virus and the plasma membrane (PM) of their host cells supports this mechanism (see section **1.5.3**). In the case of some enveloped animal viruses (rhabdo-, orthomyxoviruses, etc.), the driving force for budding is not provided by the viral core, but rather by binding of matrix proteins (Nayak et al. 2004; Solon et al. 2005).

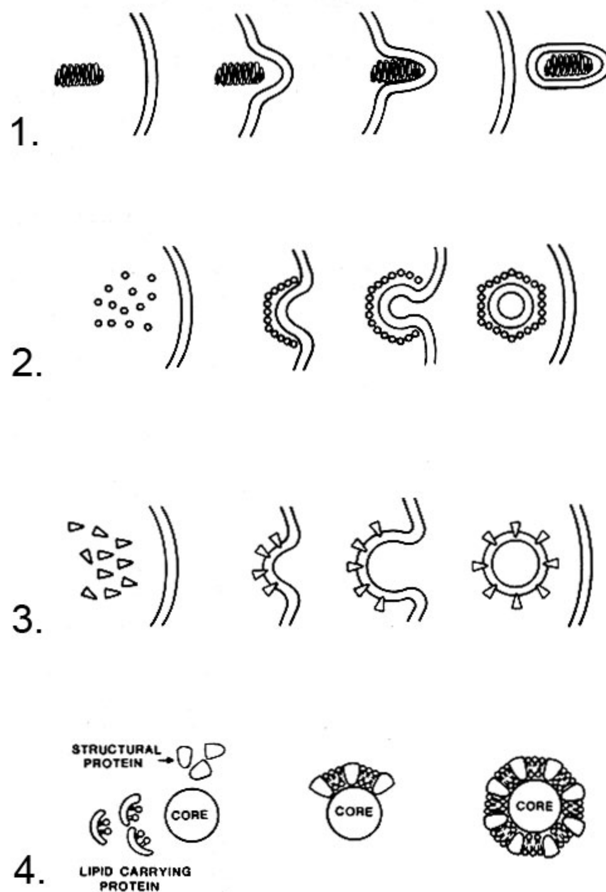

Figure 3. Different mechanisms of membrane morphogenesis. Picture redrawn from Brewer (1980).

Another mechanism (model 2) describes the formation of the viral membrane inside a protein shell (coat). In this case the coat proteins have an intrinsic property to assemble into an icosahedral shell. These proteins may also have preference for certain PLs, in which case the PL composition of a daughter membrane would be different from that of the parental one. Association of shell proteins with each other and with the membrane (e.g., membrane proteins) is a driving force for this type of membrane morphogenesis. The new membrane will form on the side of the parental membrane where the morphogenesis started, i.e., the inner leaflet of the viral membrane derives from the outer leaflet of the parent membrane (and vice versa). Clathrin-coated vesicles are probably the best known biological membrane formed via this mechanism (Pearse et al. 2000; Scales et al. 2000). The life-cycle of bacteriophage PRD1 also includes an empty capsid formed from coat proteins with a lipid membrane enclosed (see section **1.8.1.1**), indicating that phages of the *Tectiviridae* family obtain their membrane via this mechanism. Among eukaryotic viruses that have an inner lipid membrane, *Phycodnaviridae* (such as Paramecium bursaria chlorella virus-1) also seem to obtain their membranes through this pathway (Yamada et al. 2006).

In the third model (model 3), insertion of the proteins induces expansion of the inner membrane leaflet and subsequent inward curving of the whole parent membrane. Therefore viral membrane vesicles have the reverse membrane topology as compared to the host membrane. The shape and the number of copies of inserted protein would determine the size of the viral membrane, and the PL composition might partially depend on the specificity of inserted proteins to certain lipids. The insertion of caveolin into the plasma membrane of eukaryotic cells seems to induce formation of caveolae by this mechanism (Parton et al. 2006; Parton and Simons 2007). A similar vesicular intermediate within the cell has been also observed in the case of bacteriophages PM2 and phi6 (Brewer 1979; Stitt and Mindich 1983a; Johnson and Mindich 1994b), suggesting that their membranes might be formed according to this mechanism as well (sections **1.8.1.3** and **1.8.2**). In the case of phi6, which has an outer lipid envelope, the mechanism of nucleocapsid envelopment is not understood. The envelope of coronaviruses is also formed by insertion of its envelope proteins into the intracellular membrane as in this model, but the coronavirus is released to the other side of the parent membrane (Vennema et al. 1996).

According to the model 4, the viral membrane is generated *de novo* by adding viral membrane proteins and phospholipids onto a preformed core. The transfer of phospholipids could be accomplished by unknown lipid carrier proteins that may have specificity for certain lipids, thus determining the lipid composition of the new membrane. It has been initially proposed that a member of the poxvirus group, vaccinia, obtains its membrane by such *de novo* synthesis mechanism (Dales and Mosbach 1968; Stern and Dales 1974), but later it has been shown that this virus derives its membrane from a special compartment of the host endoplasmic reticulum (Sodeik and Krijnse-Locker 2002). Also the membrane assembly of bacteriophage phi6 was suggested to involve the action of a non-structural viral protein as a lipid carrier (Stitt and Mindich 1983a), but this mechanism for phi6 membrane assembly has been dismissed (section **1.8.2**). Thus the model of *de novo* synthesis of viral membrane is still a hypothetical one.

1.4 Selection of lipids to bacteriophage membranes

The PL compositions of all bacteriophages that have been studied so far were different from those of their host membranes (Braunstein and Franklin 1971; Sands 1973; Wong and Bryan 1978; Davis et al. 1982, but see also *Results and Discussion*). Specifically, the phage membranes contained relatively more PG and less PE than their hosts. Therefore, it seems that, despite the structural differences and the mechanism of membrane assembly of the phages, enrichment of PG in their membranes is a common phenomenon. Several possible mechanisms for such enrichment of PG in the bacteriophage membranes are discussed below.

1.4.1 Lipid synthesis and degradation

One explanation could be that viral infection increases the synthesis of PG or degradation of PE thus elevating the level of PG in the host membrane from which progeny virions are derived. Some animal viruses alter the synthesis of cellular lipids upon infection (Blair and Brennan 1972; Jerkofsky and De Siervo 1986; Schimmel and Traub 1987). This mechanism has also been proposed for bacteriophages to explain the increased PG content in the membranes PM2, PR4 and phi6 (Braunstein and Franklin 1971; Sands and Cadden 1975; Sands and Lowlicht 1976). However, this option of PG enrichment has been dismissed for bacteriophages PM2 and PR4 (Diedrich and Cota-Robles 1976; Muller and Cronan 1983), suggesting that other factors drive the selection of PLs to the viral membranes.

1.4.2 Budding through the PG-rich lipid domains of the host membrane

The phages could selectively obtain "patches" of host membranes that are enriched in PG. This would require that phages or their components recognize these PG-rich domains within the bacterial membranes and assemble these domains into the virion. Some animal viruses seem to use this strategy. It has been shown that the envelopes of HIV-1 and influenza are derived from the sphingomyelin- (SM) and cholesterol-rich membrane domains containing viral membrane proteins, from which cellular proteins are excluded (Suomalainen 2002; Chazal and Gerlier 2003; Suzuki and Suzuki 2006). There is no evidence, however, that domains rich in PG would exist in bacterial membranes, despite the attempts that have been made to prove their presence (Vanounou et al. 2003).

1.4.3 Lipid-protein interactions

Possibly, viral proteins inserted into the host membrane may preferentially bind PG which would result in the enrichment of this lipid in the viral membrane. Even if the affinity of viral membrane proteins for PG is similar to that of host proteins, selection of PG to the viral membrane would occur if the protein density in the viral membranes is higher than in the host membrane. Currently, little is known about the density of membrane proteins in the viral membrane or their specificity for PG, and therefore it is difficult to estimate to what extent such specific lipid binding could account for PG enrichment in the phage membranes.

Alternatively, association of the capsid proteins with the host membrane could enrich PG in the virion, since it is known that membrane-interacting domains of many peripheral membrane proteins bind negatively charged lipids via positively charged amino acid residues (Arbuzova et al. 2000). Recent structural studies on bacteriophages PM2, PRD1 and Bam35 indicated that capsid proteins of these viruses are in close association with the viral membranes (Abrescia et al. 2004; Cockburn et al. 2004; Huiskonen et al. 2004; Laurinmäki et al. 2005). Some of these viral coat proteins seem to form contacts with the viral bilayer

and can even be cross-linked with PG, but not with PE (Davis and Cronan 1985). By these interactions, PG could become enriched in the leaflet facing the protein capsid (outer leaflet in PRD1, Bam35 and PM2). However, previous studies on PM2 and PRD1 have shown that the PL composition of these viruses can be altered by changing the availability (ratio) of PLs with certain physicochemical properties in the host membranes (Tsukagoshi et al. 1975; Muller and Cronan 1983), suggesting that lipid-protein interactions may not be critical for the enrichment of PG in these viruses.

1.4.4 Shape and charge of phospholipids

It is also possible that matching the physicochemical properties, e.g. charge or effective shape, of phospholipids with the high curvature of viral membranes would be responsible for enrichment of PG in bacteriophages. The structural properties of PL molecules are, for most part, determined by the type of PL headgroup (size, charge) and fatty acids (short vs. long, saturated vs. unsaturated). Thus, depending on the headgroup and fatty acids, phospholipids have different effective shapes (see **Figure 4**).

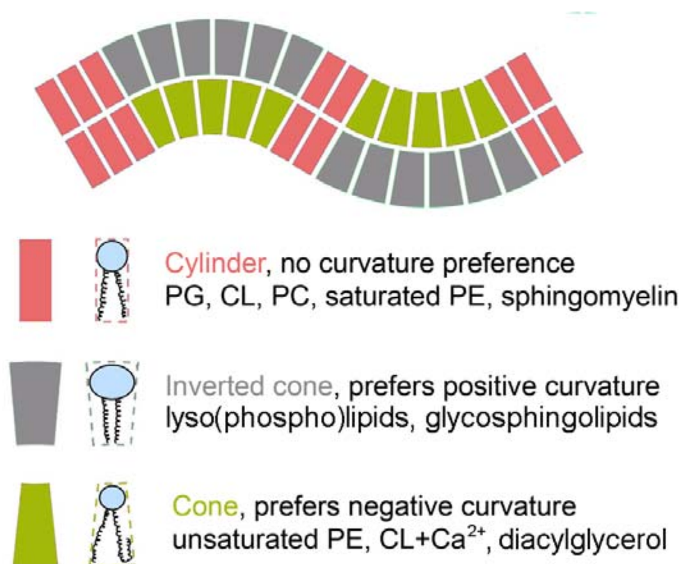

Figure 4. Effective shapes and curvature preference of (phospho)lipids.

Phospholipids with similar cross-sectional areas of the headgroup and hydrophobic tails have the shape of the cylinder. PE with saturated fatty acids, PG and CL normally have the shape of a cylinder. Lipids of this shape do not induce membrane curvature neither they do prefer any curvature due to packing constraints. Therefore such molecules form a bilayer. In contrast, phospholipids with a small headgroup are cone-shaped and thus preferably localize in the leaflet with negative curvature or even induce local curvature when inserted into a flat membrane. Unsaturated PE molecules, CL-Ca^{2+} and PG in high salt environments have the shape of a cone. Thus they would prefer the inner leaflet of the highly curved viral

membranes (Israelachvili et al. 1980). Analogously, lipids with a large headgroup (e.g., lysolipids, glycolipids etc.), would tend to be located in the outer leaflet.

Negatively charged lipids (e.g., PG and CL) repel each other due to Coulombic forces. Therefore their packing in the inner leaflet of a highly curved membrane is energetically less favorable than in the outer one due to higher charge density on the surface of the inner leaflet (Israelachvili 1973). Consequently, PG and CL should prefer the outer leaflet of the viral membranes. Due to their different effective shape and charge repulsion, phospholipids should distribute asymmetrically in the phage membranes with PG being enriched in the outer leaflet and PE preferentially localized in the inner one. Consequently, the overall enrichment of PG in the viral membranes could result from the larger surface area of the outer leaflet versus the inner one.

1.5 Lipid distribution in biological membranes

1.5.1 Lateral distribution of lipids

The classical fluid-mosaic model of cellular membranes introduced by Singer and Nicolson (1972) proposes that proteins are randomly distributed and diffuse freely in a homogeneous phospholipid bilayer, which is in a liquid-disordered phase. However, it is now well established that biological membranes are quite heterogeneous in terms of lateral distribution of proteins and lipids.

It has been shown that the membranes insoluble in cold non-ionic detergents (e.g., Triton X-100) are enriched in saturated SM and phosphatidylcholine (PC) species and contain significant proportions of cholesterol (Yu et al. 1973; Brown and Rose 1992; Koumanov et al. 2004). These detergent resistant membranes (DRMs) were suggested to represent patches of liquid-ordered domains that float as "*rafts*" within a liquid-disordered lipid bilayer (Simons and Ikonen 1997). Formation of rafts was proposed to enhance signaling cascades, since the DRMs were also enriched with signaling proteins (Schroeder et al. 1994; Melkonian et al. 1999). However, it has been observed that Triton itself promotes domain formation (Heerklotz 2002) and it is usually difficult to detect domain formation in membranes unperturbed by Triton (Munro 2003). Therefore questions were raised whether the existence of DRMs prove the presence of rafts in membranes (Lichtenberg et al. 2005). According to refined models, rafts are small (consisting of a few to hundreds of molecules), highly dynamic and unstable microdomains that can cluster within the bilayer plain into larger domains upon cross-linking of raft components or via inducing their aggregation by other means (Maxfield 2002; Edidin 2003; Subczynski and Kusumi 2003). Novel techniques are probably needed to unambiguously demonstrate the presence of rafts in cellular membranes.

Another model of lateral organization, the *superlattice model,* proposes that phospholipids are not distributed randomly in the plane of the bilayer, but adopt regular

(superlattice-like) distributions (Somerharju et al. 1999; Chong and Sugar 2002). Superlattices can form only at a limited number of allowed or "critical" lipid compositions, which *i*) allow the tightest packing of the lipids, *ii*) avoid coulombic-repulsion by charged lipids, and *iii*) increase rotational freedom of the lipid head groups (Somerharju et al. 1999). The PL compositions of both leaflets of erythrocyte membrane fall very close to the values predicted by the superlattice model (Virtanen et al. 1998), thus supporting the feasibility of superlattice formation in biological membranes. Superlattice formation has also been suggested to 1) activate/inhibit house-keeping phospholipases or phospholipid synthases thus contributing to cellular PL homeostasis, 2) assist in maintaining organelle boundaries (e.g., between ER and Golgi), and 3) dictate the lipid composition of rafts (Somerharju et al. 1999; Chong and Sugar 2002).

1.5.2 Transverse distribution of lipids

Soon after the proposal of the fluid-mosaic model for the organization of lipid membranes, the asymmetric distribution of phospholipids across the red cell membrane was discovered (Bretscher 1972a). Certain PL classes were found to be more prevalent in one leaflet of the membrane, and others in the opposite leaflet. Transbilayer asymmetry of PLs has been demonstrated in various prokaryotic and eukaryotic cells, organelles and other membrane systems (reviewed in Etemadi 1980; Roelofsen 1982; Rottem 1982).

1.5.3 Lipid asymmetry in various membranes

Plasma membranes of eukaryotic cells. After Bretscher's pioneering observation, the asymmetric transbilayer distribution of PE and other PLs was found in other studies that employed different techniques to selectively modify lipids (Gordesky et al. 1972, 1973; Verkleij et al. 1973). Erythrocytes have been studied most, because they are easy to obtain in highly pure form and the experiments are not compromised by the presence of intracellular membranes. It is now well established that a majority of the PC, SM and glycosphingolipids are located in the outer leaflet, while almost all PS and the majority of PE, various phosphatidylinositols (PI, PIP, PIP_2) and PA are located in the inner leaflet of the human erythrocyte plasma membrane (Rothman and Lenard 1977; Etemadi 1980; Roelofsen 1982; Zachowski 1993, see **Figure 5**).

The transbilayer PL distributions in the PM of erythrocytes from other species are slightly different from that of the human erythrocyte PM. For example, in mouse erythrocytes the distribution of PC is symmetrical, and all PS resides in the inner leaflet (Rawyler et al. 1985), while in erythrocytes derived from rat all SM is in the outer leaflet (Renooij et al. 1976; Crain and Zilversmit 1980, see **Figure 5** for comparison).

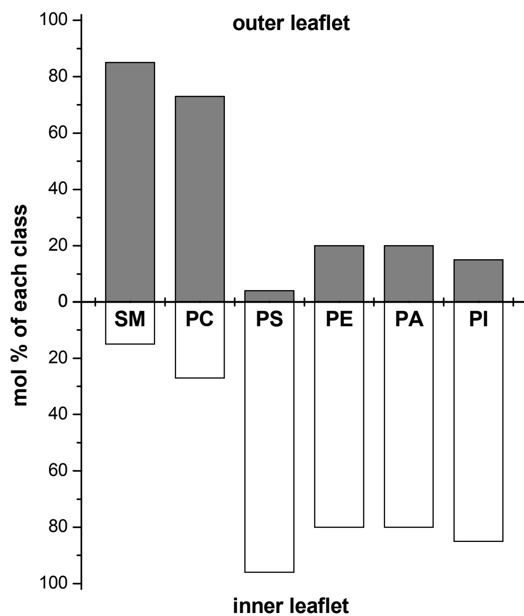

Figure 5. Transbilayer phospholipid distribution in the plasma membrane of human erythrocytes. The bars represent the fraction of each PL class in the inner and outer leaflets. The PI bar represents the averaged distribution of PI, PIP and PIP_2. The picture was drawn based on the data summarized in Zachowski (1993) and references therein.

Analysis of transbilayer PL distributions in the plasma membranes of other cell types, e.g., platelets, intestinal brush border cells, hepatocytes etc., showed that in most cases the asymmetry of SM was very similar to that of the erythrocyte PM with the exception of Krebs II ascites cells where it was distributed symmetrically (Record et al. 1984). The majority of PC, on the other hand, could be located either in the inner or in the outer leaflet, depending on the cell type (Zachowski 1993). In most cases, PE was enriched in the inner leaflet, but in some cases at least half of this lipid was located in the outer leaflet (Higgins and Evans 1978; Sessions and Horwitz 1983; Rawyler et al. 1985). PS is in each case localized largely (55% to 100% of the total PS) in the inner leaflet of the PM.

Transbilayer distribution of cholesterol, which comprises ~25% of total lipids of the mammalian PM, is controversial. One would expect that it is preferentially located in the outer leaflet (van Helvoort and van Meer 1995) due to its specific interaction with SM (Ramstedt and Slotte 1999). In some cells, however, cholesterol is suggested to be enriched in the inner leaflet of the PM (Schroeder et al. 1995), in contrast to its predicted localization.

Intracellular membranes. Despite the difficulties to prepare fractions of pure and intact subcellular organelles, the membranes of these were also shown to be asymmetric in many cases, but the distribution of different PL classes varied depending on the organelle (Etemadi 1980; Roelofsen 1982; Zachowski 1993). For instance, in sarcoplasmic reticulum of rabbit muscle SM was enriched in the lumenal leaflet, whereas in the endoplasmic reticulum from rat liver it was enriched in the cytosolic leaflet; the opposite was true for PE (Bollen and Higgins 1980; Herbette et al. 1984). In some cases controversial results were obtained. For instance, by using phospholipase A and anti-CL antibodies it was shown that CL is enriched

in the inner leaflet of the inner membrane of beef heart mitochondria (Krebs et al. 1979). However, in another study it has been suggested that ~57% of this lipid is located in the outer leaflet as judged from the specific binding of fluorescent dye 10-N-nonyl acridine orange (Petit et al. 1994). It was also shown that the distribution of CL depended on the fluctuations of ATP synthesis and inhibition of mitochondrial synthesis. More recently it was shown that the transbilayer distribution of CL in the yeast mitochondria might change quite radically upon shifting from the fermentative to the gluconeogenic growth state (Gallet et al. 1997). Those data suggest that varying distributions of phospholipids in different organelles probably reflect the specific functions of those organelles.

Bacterial membranes. In order to study membrane asymmetry in Gram-positive bacteria, one would need to digest the peptidoglycan layer with lysozyme to expose the CM for the phospholipases. Quite extensive studies employing various phospholipases and chemical reagents to modify phospholipids in the protoplasts of Gram-positive bacteria (mostly *Bacillus* species) showed that the transbilayer PL distribution is species-specific, and common principles regarding the distribution of the lipid classes could not be found. For example, PE and PG were preferentially located in the inner membrane leaflet of *Bacillus megaterium* protoplasts (Rothman and Kennedy 1977; Demant et al. 1979), while the opposite was true for *Bacillus amiloliquefaciens* (Paton et al. 1978). Moreover, the distribution of PLs in *Bacillus subtilis* could not be firmly established in two independent studies (Op den Kamp et al. 1972; Bishop et al. 1977), probably due the instability of the protoplasts. In addition, asymmetric distribution of PLs has been demonstrated for *Micrococcus lysodeikticus* (Barsukov et al. 1976), *Micrococcus luteus* (de Bony et al. 1989) and *Mycobacterium phlei* (Kumar et al. 1979), but in each case the distribution patterns were unique.

It is well established that the OM of Gram-negative bacteria is highly asymmetric with lipopolysaccharides occupying most of the outer leaflet, while the PLs are predominant in the inner one (Raetz 1978; Huijbregts et al. 2000, **Figure 1**). However, probably due to the difficulty in obtaining stable non-leaky spheroplasts, the number of studies on the transbilayer PL distribution in the CM of Gram-negative bacteria is limited. One study reported asymmetric distribution of PE in the CM and OM of Gram-negative bacterium *Erwinia carotovora* (Shukla et al. 1980). They determined that 4% of the total PE is in the external leaflet of the OM and 30% in the inner one. The outer leaflet of the CM contained 27% of total PE, whereas the remaining 38% was inaccessible to the amino group reagent, trinitrobenzene sulfonic acid (TNBS), indicating that it resides in the inner leaflet. The distribution of PG was not determined. In another study, TNBS was used to determine PE distribution in the mureinoplasts (i.e., spheroplasts with intact peptidoglycan layer) of *Alteromonas espejiana*, the host of bacteriophage PM2 (Brewer and Goto 1983). In this case, no asymmetry was found.

More recently, the transbilayer distribution of PG in the membranes of *E. coli* was studied by using sodium periodate as a modifying agent (Huijbregts et al. 1997). However, in

intact cells and in spheroplasts all of the PG was oxidized very rapidly, thus its distribution could not be determined.

Viral membranes. The membranes of several animal viruses have also been shown to be asymmetric. Two independent studies provided similar results on the lipid asymmetry in the membrane of influenza virus (Tsai and Lenard 1975; Rothman et al. 1976). PE and PS were slightly enriched in the inner leaflet and PC in the outer one. Conflicting results about the distributions of PI and SM were obtained in these two studies.

In vesicular stomatitis virus, 94% of PC, 80% of SM and 47% of PE was found in the outer leaflet (Patzer et al. 1978). The results of this study were supported by two other studies which showed that up to 70% of PC could be exchanged by a PC-specific phospholipid transfer protein (Shaw et al. 1979), whereas only ~40% of PE could be modified by TNBS (Fong et al. 1976). It was also shown that PLs with saturated FAs predominated in the outer leaflet, while unsaturated PLs were enriched in the inner one (Fong and Brown 1978; Patzer et al. 1978).

Specific and non-specific phospholipid exchange proteins, phospholipases A_2 and C, SMase and the amino agent labeling groups were used to determine the transbilayer PL distribution in the membrane of the Semliki Forest virus (van Meer et al. 1981). It was shown that PC is almost equally distributed, whereas only 22% PE and 33% SM could be allocated to the outer leaflet. In another study, however, 95% of SM, 55% of PC, 20% of PE and less then 5% of PS were found in the outer leaflet (Allan and Quinn 1989). The total PL compositions of the vesicular stomatitis and Semliki Forest viruses were similar when they were produced in the same type of cells (Patzer et al. 1978; van Meer et al. 1981; Allan and Quinn 1989). The PL asymmetries were also qualitatively similar, suggesting that the transbilayer distribution of PLs in the virus reflects that of the host cell PM.

A qualitatively similar PL distribution was also found for the membrane of Newcastle disease virus grown in embryonated chicken eggs, i.e., choline-containing phospholipids were enriched in the outer membrane leaflet, while the aminophospholipids were slightly enriched in the inner one (Suzuki et al. 1982).

Differential labeling of PE and PG has also been reported for bacteriophage PM2 (Schäfer et al. 1974, section 1.8.1.3). It suggested that the majority of PG is located in the outer leaflet of PM2 membrane, while most of PE in the inner one.

Artificial membranes. The general dogma about PL distribution in artificial membranes is that the properties of both leaflets of a flat bilayer are similar, thus PLs should eventually become randomly distributed over time. In small lipid vesicles with high radius of curvature, lipid packing is substantially different between the two monolayers. In the inner leaflet headgroup packing is tighter, whereas the acyl chains have relatively more space. In the outer leaflet, on the other hand, there is more space for headgroups, while their acyl chains become more constrained towards the center of the bilayer. Considering these differences of monolayer properties and the different physical parameters of various PL molecules into account, one would expect that different PLs would distribute differently over highly curved

bilayers according to their packing characteristics as was theoretically proposed (Israelachvili et al. 1980). The experimental evidence, however, has not always been conclusive. For instance, when the membrane asymmetry of small unilamellar vesicles composed of varying proportions of PG and PC was studied by chemical modification of exposed PG (Lentz et al. 1980), the majority of PG was found in the outer monolayer (in agreement with theoretical considerations), but the proportion of exposed PG depended on the total PG in those vesicles. However, vesicles composed of equal amounts of PG and PC showed no asymmetric distribution when studied by NMR (Nordlund et al. 1981). Moreover, later data from (Lentz et al. 1982) contradicted their earlier observations by showing that PG is distributed symmetrically in PG-PC vesicles. Contradicting data on the distribution of PE and PS has also been reported (Berden et al. 1975; Roy et al. 1997).

1.5.4 Maintenance of the phospholipid asymmetry in bilayer membranes

Transverse movement of phospholipids from one leaflet to the opposite one is called flip-flop. The half-time of PL flip-flop in the membranes of animal viruses in most cases is in the range of several hours to 30 days (Rothman et al. 1976; Shaw et al. 1979; van Meer et al. 1981), which is comparable with the half-times of PL flip-flop in vesicles, but much longer than that of protein-facilitated flip-flop in the membranes of bacteria and animal cells. This indicates that the lack of spontaneous flip-flop could maintain the PL distribution in viral membranes for a long period of time. In addition, the asymmetric distribution in viral membranes could be stabilized by lipid interaction with other lipids or with viral membrane or capsid proteins.

In biologically active membranes, e.g., erythrocyte membranes or bacterial CMs, however, the rate of flip-flop is much faster (in the order of seconds to minutes), suggesting that some proteins might actively facilitate the translocation of PL molecules from one leaflet to the other. Indeed, proteins have been identified in eukaryotic cells that not only facilitate lipid translocation across the membrane, but can also contribute to the maintenance of uneven PL distribution (for reviews see Devaux 1991, 1992; Zachowski 1993; Menon 1995; Bevers et al. 1999; Pomorski et al. 2001; Pomorski et al. 2004).

Scramblases are ATP-independent, Ca^{2+}-dependent catalyzers of bi-directional lipid translocation. Usually they are not specific for any lipid class and cannot establish an asymmetric distribution of PLs across the bilayer (reviewed in Pomorski et al. 2004; Pomorski and Menon 2006). Such proteins have been identified in eukaryotic cells (Backer and Dawidowicz 1987) and suggested to exist in bacteria also (Hrafnsdottir and Menon 2000). In contrast to nonspecific lipid translocators, phospholipid “flippases” are ATP-dependent phospholipid-specific translocases that facilitate uni-directional movement of PLs against a concentration gradient from the outer leaflet to the inner one. For instance, in the PM of eukaryotic cells, surface-exposed PE and PS are selectively translocated to the inner leaflet by aminophospholipid translocase (Devaux 1991; Daleke and Lyles 2000). Proteins

responsible for lipid translocation to the opposite direction ("floppases") have also been described. They belong to the ATP-binding cassette transporter family and can translocate PC and other PL substrates from the inner leaflet of the PM to the outer one (van Meer et al. 2006).

Upon elevation of cytosolic Ca^{2+}, the ATP-dependent translocation of PS and PE to the inner leaflet is inhibited, while the scramblase becomes activated, thus allowing translocation of more PS and PE to the outer leaflet of the cell PM. The externalized PS on the surface of thymocytes acts as an apoptotic signal of these cells which, upon recognition by macrophages, are phagocytozed (Fadok et al. 1992; Schlegel et al. 2000; Schlegel and Williamson 2001). Exposure of PS on the cell surface has been implicated in many other physiological or pathophysiological processes, such as blood clotting or sickle cell disease (reviewed in Zwaal and Schroit 1997; Schlegel and Williamson 2001).

1.6 Methods to study the transbilayer distribution of lipids

A common strategy, when studying transbilayer PL distribution, is to selectively modify the lipids on the outer leaflet of the membrane, while leaving those in the inner one unmodified. For a valid determination of transbilayer PL distribution, two aspects are critical. Firstly, the modifying agent should completely modify the lipids in the outer leaflet. Secondly, the reagent should not penetrate through the bilayer. Therefore the membrane of interest should be intact throughout the whole modification. Thus, controlling the osmolarity of the buffer solution is important. It is, however, difficult to avoid the penetration of the probe through the bilayer and the flip-flop of phospholipids from the inner leaflet to the outer one. The rate of PL modification in the outer leaflet should be much faster than that of the lipid flip-flop or reagent penetration through the membrane. To control for these issues, the reaction kinetics must be followed in order to distinguish between the modification of the outer leaflet and the inner one. Also the modification itself should not change the distribution of phospholipids. Therefore it is important to use the modifying agents that do not increase the rate of flip-flop. For instance, when phospholipase C is used to digest PLs in the outer leaflet, the diacylglycerol produced can destabilize the membrane (Bishop et al. 1977).

Lipids in the accessible leaflet can be modified by different methods. For instance, chemical reagents that specifically react with certain PLs can be utilized. The most common chemicals to study transbilayer distribution of PE and PS are reagents modifying primary amino groups, such as TNBS (Bretscher 1972b; Bishop et al. 1977; Brewer and Goto 1983), 1-fluoro-2,4-dinitrobenzene (FDNB, Gordesky et al. 1972, 1973), fluorescamine (Rawyler et al. 1984), and isethionyl acetimidate (Davis and Cronan 1985). Due to its low membrane permeability at lower temperatures (Hubbard and Cohn 1976), and relatively mild reaction conditions, TNBS has been the most used reagent.

Sodium periodate specifically oxidizes vicinal hydroxyl groups, and has been used to modify PG (as well as glycolipids) in lipid vesicles and in bacterial membranes (de Bony et

al. 1989; Hope et al. 1989; Huijbregts et al. 1997). Sulfanilic acid diazonium salt has also been used to modify PE and PG in bacteriophage PM2 (Schäfer et al. 1974).

The major drawback of chemical reagents is that, upon labeling, they may change the charge distribution within the bilayer, which could then disrupt its integrity by increasing PL flip-flop. Alternatively, introduction of bulky moieties onto the surface of the bilayer may inhibit labeling of the remaining lipids as has been observed for protoplast membranes and monolayers (Bishop et al. 1977; Bishop et al. 1979).

The inhibition due to steric crowding is avoided when the outer leaflet of the membrane is hydrolyzed by phospholipases, usually phospholipases A_2 and C. Especially phospholipase A_2 is useful to study transbilayer distribution as the products of the hydrolysis, i.e., lysophospholipids and FAs, stay in the bilayer. Phospholipases have been widely used when studying PL distribution of various membranes [e.g., erythrocytes, organelles, protoplasts of bacteria, viruses, etc. (reviewed by Roelofsen 1982)].

Enzymes can also be used to radioactively label surface components. For instance, lactoperoxidase may be used to label proteins and lipids by addition of radioactive iodine, while the galactose oxidase-sodium borohydrate method introduces tritium. By these methods, which are mainly used to label phosphoglycolipids and glycolipids, it was determined that all glycolipids are located in the outer membrane leaflet of *Acholeplasma laidlawii* (Gross and Rottem 1979).

Phospholipid transfer proteins that can exchange lipids between two membranes (Wirtz 1991) have also been used to study transbilayer distribution. Transfer proteins are particularly useful to determine transbilayer distribution of PC, since suitable chemical reagents for labeling of PC are not available. Using PC-specific transfer proteins, it is possible to determine how much of the PC, present in the target membrane, is exchangeable with (radioactive or fluorescent) PC or another lipid present in the donor vesicle by the time the PL exchange reaches plateau. The fraction of PC which is exchanged during the fast phase of the PL transfer is considered to be located in the outer leaflet (Demel et al. 1973; Demel et al. 1977). This protein was used to study the transbilayer distribution of PC in erythrocytes (van Meer et al. 1980), rat sarcoplasmic reticulum (de Kruijff et al. 1979), influenza virus (Lenard and Rothman 1976; Rothman et al. 1976), Semliki Forest virus (van Meer et al. 1981) and lipid vesicles (Rothman and Dawidowicz 1975). Also non-specific phospholipid exchange proteins and proteins specific for PI and PG have been identified and purified (for review see Wirtz 1991), and this approach is not limited only to PC.

Some physical methods have also been used to study the transbilayer distribution of phospholipids. For instance, by measuring the nuclear magnetic resonance of a given lipid before and after addition of shift reagents, it is possible to determine the transbilayer distribution of that lipid over the bilayer. This technique has been mostly used to determine lipid transbilayer distribution in artificial lipid vesicles (de Kruyff et al. 1977; Barsukov et al. 1980; Nordlund et al. 1981). Electron spin resonance and fluorescence measurements allow one to determine the transbilayer distribution of spin- or fluorescently labeled lipid analogs,

respectively. These techniques are most often used to study transmembrane movement of PLs, rather than their transbilayer distribution since the labeled lipid may not distribute equally to natural ones.

1.7 Mass-spectrometry as a tool to study lipid compositions

Electrospray ionization mass-spectrometry (ESI-MS) is a particularly suitable method to determine the PL class and molecular species compositions. In addition to saved time, ESI-MS has other advantages over the conventional methods. Firstly, MS is a sensitive method and it requires far less material than the conventional methods (Brügger et al. 1997). Secondly, it is a selective method as it is possible to detect and analyze molecules that differ in mass by ~1 dalton. This allows the simultaneous analysis of the total lipid extracts containing hundreds of different molecular species. The identity of PL molecular species can be determined by fragmentation analysis followed by the detection of the fatty acyl ions (Han and Gross 2003). Thirdly, it is a specific method, since it is possible to scan for one particular PL class from the total lipid extract. For instance, by scanning for neutral loss of 141, PE molecular species are specifically detected in the positive ion mode (Kerwin et al. 1994; Brugger et al. 1997), while scanning for precursors of 171 in the negative ion mode PG molecular species are detected (Hsu and Turk 2001).

Furthermore, by adding appropriate internal standards to the total lipid extract (at least two per each PL class), quantitative analysis of PL class and molecular species compositions can be performed (Käkelä et al. 2003). This is necessary in order to correct the instrument response for the effect of the acyl chain (Koivusalo et al. 2001). The daily use of ESI-MS produces huge amounts of data that is difficult to analyze manually. To enable high-throughput analysis special software has been developed (Haimi et al. 2006).

1.8 Structures of lipid-containing bacteriophages

Lipid-containing bacteriophages with different morphologies and localization of the lipid membrane have been isolated. They can be spherical with an icosahedral capsid (such as bacteriophages of the *Tectiviridae*, *Corticovirdae* or *Cystoviridae* families), filamentous (*Lipothrixviridae*) or pleomorphic (such as *Plasmaviridae* and *Fuselloviridae*, **Figure 6**). The most studied are icosahedral bacteriophages of the *Tectiviridae* and *Corticoviridae* families that contain an internal lipid membrane, probably due to "historical" reasons of being among the first lipid-containing phages to be identified and due to the "susceptibility" of their hosts for genetic manipulation.

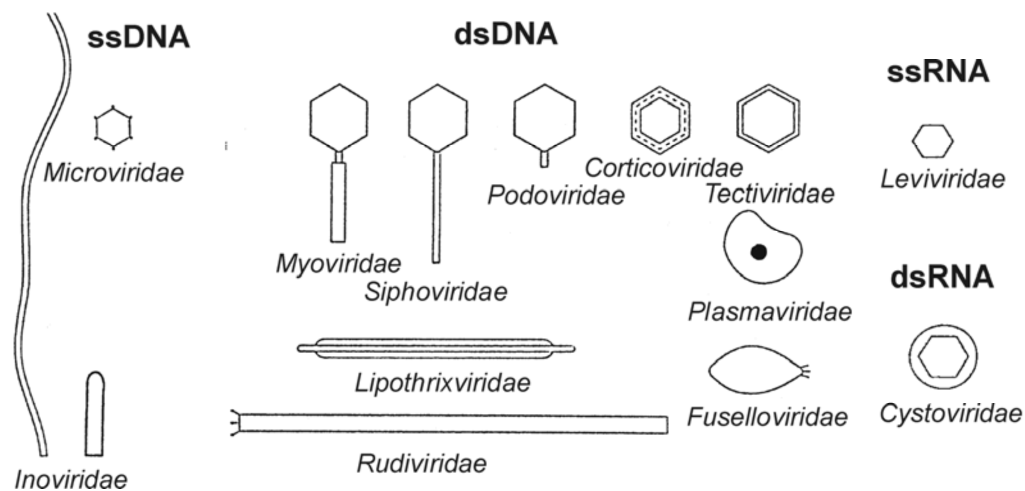

Figure 6. Morphologies of bacteriophages. Lipid-containing phages belong to *Tecti*-, *Cortico*-, *Cysto*-, *Lipothrix*-, *Plasma*- and *Fuselloviridae* families. Adapted from Ackermann (2003).

1.8.1 Icosahedral phages with an internal membrane

1.8.1.1 PRD1

Overview. The lipid-containing bacteriophage PRD1 is the type-organism of the *Tectiviridae* family. Other PRD1-related bacteriophages PR3, PR4, PR5, PR772 and L17 infecting Gram-negative bacteria (Bamford et al. 1981) and bacteriophages Bam35, AP50, phiNS11, GIL01 and GIL16 infecting Gram-positive hosts belong to the same family (Bamford and Ackermann 2000). The sequence of PRD1 is 91.9-99.8% identical to the sequences of its relatives (Saren et al. 2005) although these phages have been isolated in different parts of the world. Due to such high genomic and structural conservation, the results obtained with one virus are considered to be generally applicable to the others.

PRD1 is an icosahedral double-stranded DNA virus infecting a broad range of Gram-negative bacteria, such as *Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa*, that contain an IncP, IncN or IncW conjugative plasmid (Bamford et al. 1995a; Grahn et al. 2003). This property of tectiviruses infecting Gram-negative hosts has been used to advantage when studying membrane structure and morphogenesis in a simple model system, because it was possible to propagate viruses in the host cell mutants of *E.coli* defective in the synthesis of various phospholipids and FAs.

Methods for purification of PRD1 in large quantities have been described (Bamford et al. 1995b), and have allowed extensive biochemical and structural characterization of the whole virus and its components (Caldentey et al. 1990; Bamford et al. 1995a; Butcher et al. 1995; San Martin et al. 2002a; Grahn et al. 2006). Crystals of PRD1 have been obtained (Bamford et al. 2002b; Cockburn et al. 2003), which allowed the determination of its structure by X-ray crystallography (Abrescia et al. 2004; Cockburn et al. 2004). The

observed similarity of the capsid architecture and the fold of the major coat proteins between PRD1 and adenovirus suggested that these two viruses might be related, i.e., belong to the same viral lineage, despite very distant hosts (Benson et al. 1999; Bamford et al. 2002a; Bamford 2003). This hypothesis prompted the search of new viruses that would share the same capsid architecture and the fold of the major coat protein, but infect organisms of other domains of life (Ravantti et al. 2003; Bamford et al. 2005; Khayat et al. 2005; Maaty et al. 2006).

Architecture of the virion. The PRD1 particle (**Figure 7,** panel A) is approximately 640Å in diameter and the outermost protein capsid is composed of trimeric major coat protein P3, the 240 copies of which are organized in a pseudo T=25 lattice (Butcher et al. 1995; San Martin et al. 2002b). The capsid proteins of adenovirus are also organized into a pseudo T=25 lattice and share the same fold (two beta-barrels) as that of PRD1 (Benson et al. 1999).

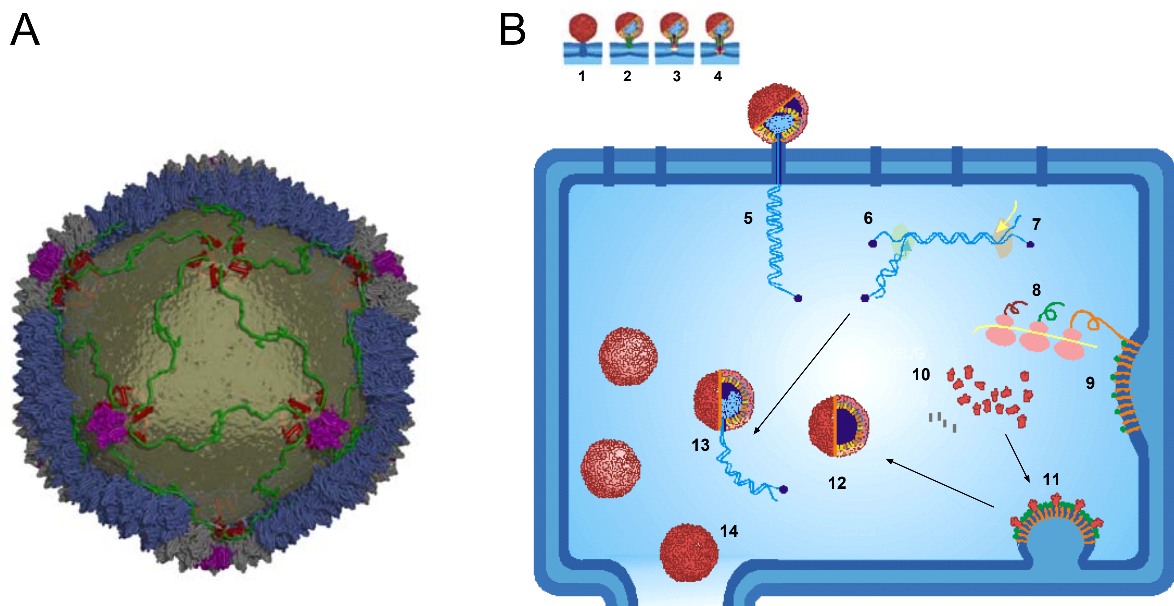

Figure 7. **A**) The structure of PRD1 determined by X-ray crystallography. The P3 trimers (blue and grey) are partly removed to show the membrane (yellow) and protein P30 (green) connecting the vertices, where pentameric P31 (magenta) resides. The spikes (composed of P5 and P2) are not shown. Integral membrane protein P16 (red) links the spike complex to the membrane and is necessary for stability of the spike (Jaatinen et al. 2004). The 14927 bp long double-stranded DNA molecule with terminal proteins is packaged within the membrane vesicle (not shown). Adapted from Cockburn et al. (2004). **B**) Life-cycle of PRD1: 1) receptor binding, 2) OM penetration, 3) peptidoglycan digestion, 4) fusion with CM, 5) DNA injection, 6) protein-primed replication, 7) transcription, 8) translation, 9) insertion of viral membrane proteins into host CM, 10) folding of synthesized coat proteins in the cytosol, 11) association of coat proteins with the host CM, 12) procapsid formation, 13) DNA packaging, 14) lysis. Adapted from Grahn et al. (2006).

In PRD1, the minor capsid protein P30, which is required for particle assembly (Rydman et al. 2001), lies elongated below the P3 layer. At the vertices of the capsid there

are spike complexes, via which PRD1 binds to the cell surface receptor (Bamford and Bamford 2000; Caldentey et al. 2000). One of the vertices composed of small membrane proteins P20 and P22, packaging factor P6 and packaging ATPase P9 is unique in the way that it contains a portal structure through which the genome is packaged in an ATP-dependent manner (Gowen et al. 2003; Strömsten et al. 2003). Beneath the protein capsid there is an internal membrane as seen in negative-staining electron micrographs of empty PR4 particles (Lundstrom et al. 1979). It is composed of virally encoded membrane proteins (at least 10) and lipids derived from the host cell. The viral genome is packaged within this membrane vesicle.

Life-cycle. The life-cycle of PRD1 is depicted in **Figure 7** (panel B). PRD1 recognizes the host cell surface receptor, encoded by a conjugative plasmid, via protein P2 located in the spike complex. Upon the receptor recognition, the vertex structure becomes unstable and disassembles. This triggers a cascade of events that lead to the delivery of the DNA into the cytosol. Membrane proteins P7, P11, P14, P18 and P32 are involved in this process. P7 acts as a lytic transglycosylase to allow penetration through the peptidoglycan layer. Particles devoid of this protein are infectious, but the delivery of DNA into the cytosol is prolonged (Rydman and Bamford 2000). Membrane protein P11 has been suggested to act first in the cascade of DNA delivery, since the infection of cells with $P11^{-}$ mutant was not able to increase the permeability of the OM to lipophilic compounds, in contrast to wt PRD1 particles (Grahn et al. 2002a). Proteins P14, P18 and P32 are necessary for the transformation of the viral membrane into a tube-like structure, which has been suggested to penetrate the cell envelope and inject the viral DNA into the cytosol (Lundstrom et al. 1979; Bamford and Mindich 1982). The absence of any of these proteins abolishes tube formation (Grahn et al. 2002a, b). Observations that certain FAs and their derivatives interfere with the infection, but not with the attachment of PR4 virions to the host cell (Reinhardt et al. 1978; Sands et al. 1979), are in line with the involvement of the membrane in the DNA delivery process. Similar tubes were also observed in the case of bacteriophages PR5 (Wong and Bryan 1978), and Bam35 (Ackermann et al. 1978; Laurinmäki et al. 2005).

The replication of PRD1 genome is carried out by the phage-encoded DNA polymerase (Savilahti et al. 1991). Mg^{2+} ions are needed for activation of this enzyme (Caldentey et al. 1992). The terminal proteins (P8) attached to 5' ends of the DNA molecule act as primers for replication (Savilahti et al. 1989). Some other phages and adenovirus also use a similar mechanism to prime replication of their genomes (Salas 1991).

After transcription and translation, the major coat protein (P3) and the proteins involved in the spike-complex (P2, P5 and P31) are found soluble in the cytosol, and the membrane proteins of the phage are inserted into the host CM (Mindich et al. 1982). This patch of host CM rich in viral proteins associates with the forming viral capsid (composed of P3) with the help of non-structural phage-encoded assembly factors (P10, P17, and possibly P33). In addition, minor coat protein P30 is necessary for this association since cells infected with a $p30^{-}$ mutants produce virus-specific membrane vesicles containing reduced amounts of P3

(Rydman et al. 2001). When the association of the major coat protein with the membrane patch is completed, particles consisting of the viral membrane and the protein capsid, but devoid of viral DNA (procapsids) are formed. The above model of membrane morphogenesis in PRD1 is in line with model 2 described above (**Figure 3,** section **1.3**), and suggests that the outer leaflet of the viral membrane is derived from the inner leaflet of the host CM (and vice versa). Cryo-EM studies on empty PRD1 particles (*sus1* mutant) showed that the viral membrane was not spherical, but followed the inner surface of the icosahedral protein capsid with which it made several contacts (Butcher et al. 1995). The membrane is pushed towards the capsid and more contacts between them are formed when the procapsids are packaged with DNA. Some PLs may even be cross-linked to the major coat protein (Davis and Cronan 1985).

The packaging reaction is carried out by packaging ATPase P9 (Strömsten et al. 2005). This enzyme is a structural viral protein and a component of the portal complex located at the unique PRD1 vertex (Strömsten et al. 2003). It has been shown by Raman spectroscopy that the packaged DNA extensively interacts with the membrane, possibly with the PE headgroups (Tuma et al. 1996). The mature virions are released from the cell by host cell lysis. This process involves the action of phage-encoded endolysin, which is released to the periplasm by the aid of holins, small integral membrane proteins that form lesions in the host CM (Rydman and Bamford 2002, 2003; Žiedaitė et al. 2005).

Viral membrane. Approximately 40% of the viral membrane mass consists of proteins, and the rest is lipids (Davis et al. 1982). The first analysis of PR4 virion lipids showed that it contains PG, PE, CL, PS and an unknown lipid species (Sands and Cadden 1975). The quantitative differences between the viral and host PL compositions were shown not to be due to a change of PL synthesis upon infection (Sands 1976). The presence of PS and unknown lipids was later dismissed by another groups, which found that bacteriophage PR4 produced in *E.coli* TD6 cells contained only PE, PG and CL representing 56, 37 and 4.5% of the total PLs, respectively (Davis et al. 1982). The FA composition resembled that of the *E. coli* host. Bacteriophage PR5 produced in *E.coli* K12 cells also contained only PE, PG and CL in proportions similar to those of PR4 grown in the TD6 strain (Wong and Bryan 1978). In both cases, PG was enriched and was PE depleted in the viral membrane as compared to the host.

The effects of altered PL compositions on the production of PR4 virions have been studied by propagating virions in *E.coli* mutants defective in the synthesis of either fatty acids or phospholipids (Muller and Cronan 1983). In response to the PL composition changes within the host, PR4 particles with PL composition varying from 28 to 60% in PE, from 22 to 39% in PG, from 2 to 15% in CL and from <0.5 to 35% in PS were produced. These changes in the PL composition of the virion did not affect infectivity. Interestingly, in all cases PG was enriched in the viral membrane and PE depleted to the same extent as in virus produced in a wild-type host (Muller and Cronan 1983). This suggested that lipids are not specifically bound to the phage proteins nor is the charge of viral PLs critical for the enrichment of PG in

the viral membranes. Instead, it was proposed that the viral membrane might be derived from patches of the host CM rich in PG. However, no evidence exists to support this idea. Moreover, it has been shown that the enrichment of PG is not essential for the assembly of PR4, as infectious virions with only ~0.5% PG could be produced (Vanden Boom and Cronan 1988). In this case, the PA probably substituted for PG, as its content rose from 4.5% in the host to ~10% in PR4. Bacteriophage PR4 incorporates PI, monoacylglycerol and diacylglycerol when they are present in the host membrane (in small amounts), but avoids incorporation of unnatural lipids with bulky polyol headgroups (Myung and Cronan 1994), thus suggesting that the latter lipids are not compatible with the structure of the viral bilayer and lipid packing therein.

Lipids in wild-type PRD1 are in liquid crystalline phase between 5 and 50 ºC, as shown by Laser Raman spectroscopy (Tuma et al. 1996). By infecting temperature-sensitive cells incapable of the synthesis of unsaturated FAs, PR4 with various ratios of saturated versus unsaturated FAs were obtained. It was observed that increasing the proportion of saturated FAs in the virion diminished its stability during the purification storage (Muller and Cronan 1983), indicating that large amounts of saturated FAs, which increase the order in the membrane bilayer, are not compatible with the integrity of the PR4 membrane.

Cryo-EM studies provided insights into the interactions between the lipid membrane and the viral capsid in PRD1 (Butcher et al. 1995; Martin et al. 2001). Determination of the virion structure by X-ray crystallography allowed more detailed characterization of those interactions and indicated possible asymmetric distribution of phospholipids (Bamford et al. 2002b; Cockburn et al. 2003; Abrescia et al. 2004; Cockburn et al. 2004, see Results and discussion).

1.8.1.2 Bam35

The first description of bacteriophage Bam35 infecting *Bacillus thuringiensis* appeared in late 70's (Ackermann et al. 1978). It was after the proposal that viruses form structure-based lineages (Benson et al. 1999; Bamford et al. 2002a; Bamford 2003), when further studies on this bacteriophage were initiated. Bam35 appeared morphologically indistinguishable from PRD1, and its double-stranded DNA genome had very similar length and is probably covalently linked to terminal proteins as in PRD1 (Ravantti et al. 2003). Also the organization of Bam35 genome is very similar to that of PRD1 despite the lack of significant sequence similarity between the two viruses. Thirty-two open reading frames were suggested to code for proteins. Ten of the 32 putative genes encoded one predicted transmembrane helix, suggesting that these gene products are membrane –associated (Ravantti et al. 2003). The major coat protein of Bam35 has the same fold as that found in PRD1 (Ravantti et al. 2003; Benson et al. 2004), thus supporting the idea that these two viruses belong to the same lineage.

The DNA molecule of Bam35 is enclosed within a membrane vesicle, which is further surrounded by an icosahedral protein capsid. As shown by cryo-EM (Laurinmäki et al. 2005),

in intact virions the protrusions of the major coat protein contact the membrane, pressed against the capsid upon packaging of DNA. The overall structure of Bam35 capsid resembles that of PRD1, but the greatest differences between the two viruses lie in the structure of their membranes. The membrane of Bam35 was found thinner than that of PRD1, and the membrane curvature profiles were clearly different. The areas of higher curvature in Bam35 membrane coincided with the position of the complex of transmembrane proteins, indicating that they modulate the structure of the viral membrane (Laurinmäki et al. 2005).

Bam35 adsorbs to the peptidoglycan of the host cell, *N*-acetyl-muramic acid acting as a „receptor". There is a peptidoglycan-hydrolyzing activity associated with Bam35 virions, which probably aids the delivery of the genome into the host cytoplasm (Gaidelytė et al. 2006).

The membrane of PRD1 is able to transform into a tubular structure, implicated in the delivery of PRD1 genome to the host cytoplasm, while leaving empty capsids on the surface of the cell (Grahn et al. 2002a, b). Similar membranous tubes are occasionally observed in Bam35 (Ackermann et al. 1978; Laurinmäki et al. 2005), suggesting that it is involved in the genome delivery to the host cell, despite different cell wall structures of the Gram-negative and Gram-positive host bacteria. Upon genome delivery, the CM of the Bam35 host remains practically intact as in the case of PRD1 (Gaidelytė et al. 2006), supporting the idea that the membranes of Bam35 and its host interact (probably fuse). Depending on the host strain, Bam35 may enter either a lytic or a lysogenic replication pathway. In the latter case, the genome of Bam35 remains in the host as a ~15kb plasmid and the expression of most viral genes is suppressed (Gaidelytė et al. 2005). In the case of the lytic pathway, however, the mature progeny virions are produced within the host cytoplasm ~40 min post-infection. They are released from the cell upon cell lysis which is possibly induced by the holin-endolysin system, similar to that of PRD1, as indicated by the premature lysis of Bam35-infected cells occurring after addition of metabolic inhibitors (Gaidelytė et al. 2005).

1.8.1.3 PM2

Overview. Bacteriophage PM2, isolated from the seawater at the coast of Chile together with its Gram-negative host *Pseudoalteromonas espejiana* BAL-31 [previously named *Pseudomonas* BAL-31 or *Alteromonas espejiana* BAL-31 (Espejo and Canelo 1968)], has become an attractive biological system due to two unique characteristics. First, PM2 was the first lipid-containing bacteriophage identified, and thus provided a model system to study membrane structure and morphogenesis. Second, the genome of PM2 is a highly supercoiled circular double-stranded DNA molecule, which has been widely used in DNA topology studies. PM2 is the only isolated member of the *Corticoviridae* family, but numerous putative prophages closely related to PM2 have been identified (Krupovič and Bamford 2007). PM2 can also be propagated in *Pseudoalteromonas sp*. ER72M2 cells (Kivelä et al. 1999), which allows production of larger quantities of phage necessary for its detailed biochemical and structural characterization.

The virion of PM2 (**Figure 8, panel A**) has a diameter of ~60 nm and consists of an outer icosahedral protein capsid and an internal lipid membrane, within which the circular supercoiled double-stranded DNA genome is enclosed (Kivelä et al. 2002).

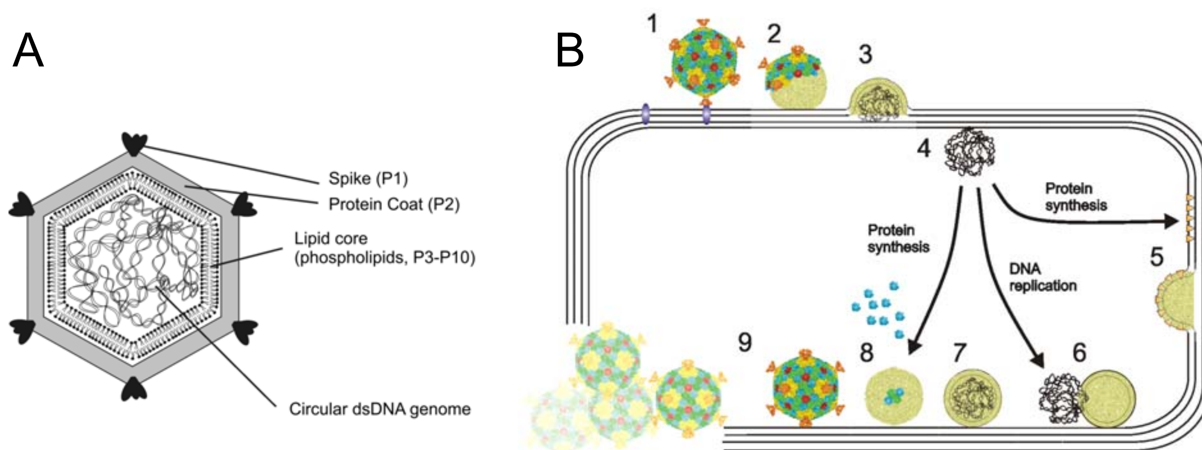

Figure 8. **A**) Schematic structure of bacteriophage PM2. **B**) Infection cycle of PM2: 1) receptor recognition, 2) disassembly of the capsid, 3) fusion of the lipid core with the host OM, 4) DNA entry, 5) insertion of membrane proteins and membrane invagination, 6) association of DNA with lipid vesicle, 7) formation of the lipid core, 8) association of coat and spike proteins to form virions, 9) lysis. Panel A is adapted from Kivelä et al. (2002) and panel B is a courtesy of Dr. Hanna Kivelä.

Protein constitutes ~72%, nucleic acid ~14% and lipid ~14% of the estimated ~45 MDa mass of the virion (Camerini-Otero and Franklin 1975). Out of 42 open reading frames found in the PM2 genome, 21 were identified as putative genes organized in three operons (Männistö et al. 1999). At least ten of these genes were identified as the structural proteins of the PM2 virion (Kivelä et al. 1999; Kivelä et al. 2002). Proteins P1 and P2 were identified as spikes and major coat proteins, respectively, while proteins P3-P10 are membrane associated (Schäfer et al. 1974; Kivelä et al. 1999; Männistö et al. 1999; Kivelä et al. 2002; Huiskonen et al. 2004).

Lipids are organized in a bilayer, although the amount of lipids per virion is sufficient to occupy only 50-70% of the whole membrane volume (Harrison et al. 1971; Camerini-Otero and Franklin 1972). The rest of the membrane volume is probably taken up by proteins. Based on quantitative dissociation studies, it was suggested that the viral membrane (lipids and membrane-associated proteins) and the genome constitute a lipid core onto which proteins P2 and P1 assemble to form mature virions. The suggested architecture of the PM2 virion (Kivelä et al. 2002) has been confirmed by cryo-EM studies (Huiskonen et al. 2004). It seems quite similar to that of PRD1 despite the differences in genome organization.

Life-cycle. The infection cycle of PM2 (**Figure 8, panel B**) starts with the recognition of the host cell receptor by the spike protein P1, after which the viral capsid dissociates and the lipid core fuses with the host OM (Kivelä et al. 2004). Membrane-associated protein P7 has been proposed to aid the penetration of PM2 genome through the peptidoglycan layer (Kivelä et al. 2004). The genome is delivered to the host cytoplasm through a putative

protein pore in the host CM as suggested by electrochemical measurements (Kivelä et al. 2004). After this the genome is replicated using the rolling circle mechanism (Espejo et al. 1971; Männistö et al. 1999).

The replication of PM2 virions takes place close to the CM as evidenced by electron microscopy studies (Cota-Robles et al. 1968; Dahlberg and Franklin 1970; Brewer 1978). One of the intermediates in the assembly of PM2 virions could be empty virus-sized membrane vesicles that were observed upon infection of the host cells with temperature-sensitive PM2 mutants (Brewer 1976). These empty vesicles subsequently become filled with DNA to produce lipid cores. Calcium ions are required to stabilize the association of the major coat proteins P2 and the spikes with the lipid cores to form mature virions inside the host cell (Snipes et al. 1974; Kivelä et al. 1999). The virions (~300 particles per cell) are released by the host cell lysis.

Viral membrane. The membrane of PM2 consists of ~64% PG, ~27% PE, ~7% neutral lipids, and ~1% other lipids [e.g., PA, acyl-PG (Braunstein and Franklin 1971; Camerini-Otero and Franklin 1972; Tsukagoshi et al. 1976a)]. The PL composition of PM2 is different form that of its host *Pseudoalteromonas espejiana* BAL-31, which was found to contain ~25% PG and ~75% PE (Braunstein and Franklin 1971), with the CM containing slightly more PG and less PE than the OM (Diedrich and Cota-Robles 1974). It has been shown that the synthesis and turnover of PE and PG are not affected during the assembly of PM2 in infected host cells (Diedrich and Cota-Robles 1976). Consequently it was concluded that the relative enrichment of PG in the membrane of PM2 as compared to its host is not due to the increased PG synthesis, as had been suggested (Braunstein and Franklin 1971).

Based on differential labeling of PLs with sulfanilic acid diazonium salt it was proposed that PLs are asymmetrically arranged in the PM2 membrane, with PG being preferentially located in the outer leaflet and PE in the inner one (Schäfer et al. 1974). The exact proportions of these PLs located in the inner and outer leaflets are, however, not clear, since the kinetics of PL modification was not determined. In another study, the kinetics of PE labeling with TNBS in PM2-specific membrane vesicles was determined, but in contrast with the study of Schaffer et al. (1974), it was found that more than 50% of the PE resides in the outer leaflet (Brewer and Goto 1983). Thus the transbilayer distribution of phospholipids in PM2 membrane has not been unambiguously established.

FA compositions of PM2 and *Pseudoalteromonas espejiana* BAL-31 are very similar with the 16:1, 16:0 and 18:1 species being the predominant ones (Camerini-Otero and Franklin 1972). Consequently the lipid bilayer of PM2 is in a liquid-crystalline state at the physiological temperatures of its host (Tsukagoshi et al. 1976b). The FA composition of PM2 reflects that of the host when the virus is propagated in the FA auxotroph strain UFA of *Pseudoalteromonas espejiana* BAL-31. When the host cells' culture medium was supplemented with cis-16:1 or cis-18:1 fatty acids, the FA compositions of the host and PM2 produced therein were almost identical (Tsukagoshi et al. 1975). On the other hand, in the presence of trans-16:1, resembling saturated 16:0 by its properties (Chapman et al. 1966),

PM2 contained relatively more saturated FAs than the host (~11% in PM2 vs. ~1% in the host).

Interestingly, the PL class composition of PM2 changed significantly depending on the FA given to the auxotrophic host. The largest deviation from the PL class composition of PM2 produced in wt *Pseudoalteromonas espejiana* BAL-31 (~51% PE and ~42% PG vs. ~38% PE and ~58% PG) was achieved when PM2 was propagated in the UFA strain supplemented with the trans-16:1 fatty acid (Tsukagoshi et al. 1975). This result suggests that the physical properties of the PL molecules control the PL class compositions in the viral membrane via an undefined mechanism.

The production of empty vesicles, with the size, shape and lipid composition of the PM2 membrane, upon infection of host cells with PM2 mutants (ts1 and ts5) indicates that insertion of a virally-encoded membrane protein(s) into the host membrane causes invagination of that membrane and subsequent release of the vesicle into the cytoplasm. This suggests that the membrane morphogenesis of PM2 occurs as depicted in model 3 (**Figure 3,** section **1.3**). This further suggests that the viral lipids are derived from the host and that the inner leaflet of the viral membrane originates from the outer leaflet of the host CM.

1.8.2 Enveloped bacteriophages of the *Cystoviridae* family

Overview. The *Cystoviridae* family consists of the double-stranded RNA bacteriophages. Bacteriophage phi6 is the type organism of this family, but 8 members (phi7-14) have been isolated and partially characterized (Mindich et al. 1999). Phi6 infects a Gram-negative pathogen of beans, *Pseudomonas syringae pv. phaseolicola* HB10Y, while other members may also infect other Gram-negative hosts (e.g., *E.coli* or *S.typhimurium*). Phi6 has two unique features that have made it quite invaluable as a model system. First, phi6 contains a segmented dsRNA genome. Its replication appears to be similar to that of eukaryotic dsRNA viruses (Bamford et al. 2001). Second, it has a lipid envelope containing only a few virally encoded proteins (Vidaver et al. 1973). The latter phenomenon not only provides a very simple model system to study membrane structure and morphogenesis, but is also intriguing because the membrane topology is not compatible with currently known mechanisms for membrane morphogenesis (section **1.3**). Among cystoviruses, phi6 is the best characterized phage physiologically, biochemically and the structure of its virion has been determined by cryo-EM (Butcher et al. 1997)

Architecture of the phi6 virion. Based on cryo-EM studies, the phi6 virions are spherical particles with a diameter of ~86 nm (Kenney et al. 1992). A schematic representation of the virion structure is depicted in **Figure 9** (**panel A**).

The procapsid of phi6 virion is composed of viral proteins P1, P2, P4 and P7 (Bamford and Mindich 1980; Kakitani et al. 1980; Mindich and Davidoff-Abelson 1980). P1 forms the inner dodecahedral capsid (Ktistakis and Lang 1987). The hexameric packaging NTPase (P4) is located at the five-fold positions of the P1 core (Gottlieb et al. 1992; de Haas et al. 1999). Protein P7 is an assembly factor, but it is also required for packaging (Juuti and Bamford

1997; Poranen et al. 2001). P2, located beneath the five-fold vertices, is a RNA-dependent RNA polymerase which is responsible for the synthesis of a dsRNA from the single-stranded templates (Makeyev and Bamford 2000). In mature virions, the segmented dsRNA genome (l, m and s segments) is located within the procapsid, which in turn is surrounded by a porous layer of protein P8 organized in a T=13 lattice (Butcher et al. 1997).

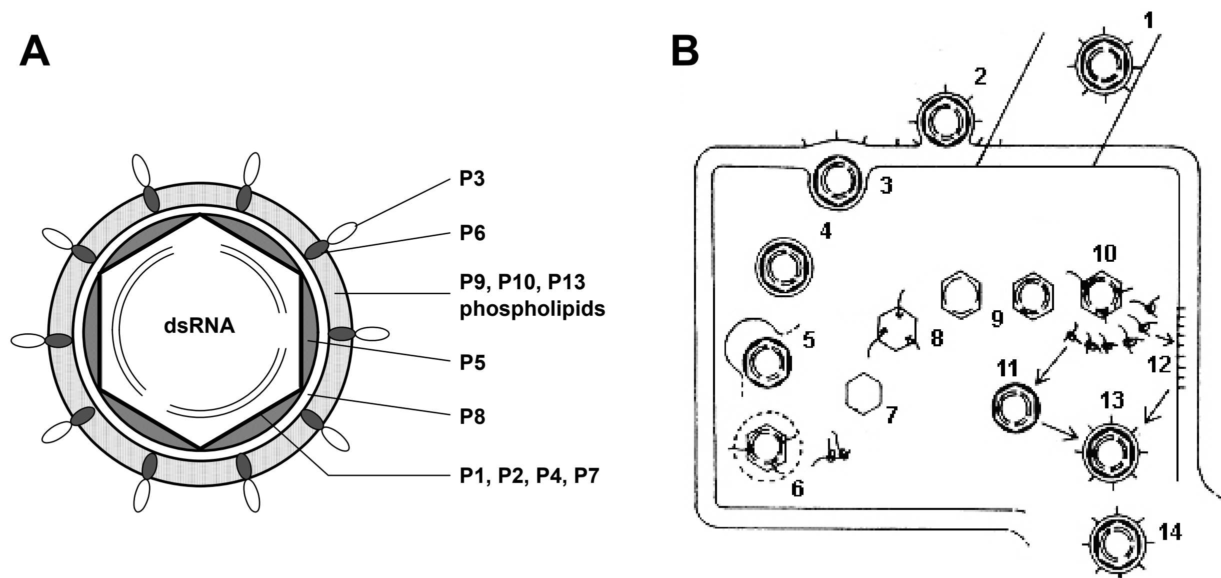

Figure 9. A) Schematic presentation of bacteriophage phi6 virions. The membrane is shown in dark yellow, spikes in light yellow and P8 layer in green. **B)** Schematic representation of the life-cycle of phi6: 1) attachment to pilus, 2) fusion with the host OM, 3) peptidoglycan digestion, 4) entry into the host, 5) nucleocapsid uncoating, 6) early transcription and protein synthesis, 7) procapsid assembly, 8) ssRNA packaging, 9) dsRNA synthesis, 10) late transcription and protein synthesis, 11) NC assembly, 12) synthesis of membrane proteins, 13) envelope assembly onto NC, and 14) lysis of host cell. Panel A is modified from (II) and panel B is taken from Olkkonen (1990).

Ca^{2+} ions stabilize the P8 shell on the surface of the nucleocapsid (Ktistakis and Lang 1987; Olkkonen and Bamford 1987). A small viral protein P5 (lytic enzyme) resides between the P8 layer and the viral membrane as indicated by cross-linking experiments (Hantula and Bamford 1988). The nucleocapsid is surrounded by the membrane, which consists of PLs (Sands 1973) and phage-encoded membrane proteins P6, P9, P10, P11 and P13 (Sinclair et al. 1975; Etten et al. 1976). The distance from the outer surface of the viral membrane to the surface of the nucleocapsid is ~6 nm (Kenney et al. 1992). The fifth membrane-associated protein P3 is attached to the protein P6 and forms spikes (Etten et al. 1976; Stitt and Mindich 1983b).

Life-cycle. The life-cycle of phi6 is presented in **Figure 9** (**panel B**). First, the phi6 virion is attached to a host pilus via its spike protein P3 (Poranen et al. 1999). Retraction of the pilus brings the phi6 particle into contact with the host OM. Viral protein P6 then promotes fusion of the viral envelope with the host OM (Bamford et al. 1990) and the nucleocapsid enters the periplasmic space of the host. After the digestion of the peptidoglycan by the viral endopeptidase P5 (Lehman and Mindich 1979; Caldentey and

Bamford 1992), the nucleocapsid comes into contact with the host CM and is subsequently endocytosed via a membrane potential-dependent mechanism (Poranen et al. 1999).

The P8 protein has been suggested to play a role in the nucleocapsid penetration through the host CM. In the absence of Ca^{2+}, P8 is unstable undergoes local conformational changes which have been implicated in the uncoating of the nucleocpasid-containing vesicle and the release of the procapsid into the cytosol (Romantschuk et al. 1988; Tuma et al. 1999). The procapsid acts as a transcriptase and synthesizes the plus-sense mRNA molecules of all three segments (Coplin et al. 1975). Early in in the infection, however, only the mRNA of the l segment is translated to produce proteins P1, P2, P4 and P7. These proteins assemble empty procapsids (Poranen et al. 2001), to which the plus-strands of all three segments are packaged in a sequential manner. Upon packaging, the procapsid expands which is accomplished by the rotation of P1 monomers (Huiskonen et al. 2006). The minus-strand is synthesized inside the procapsid (Gottlieb et al. 1990). During the late transcription mainly m and s mRNAs are synthesized, which leads to the production of protein P8 and membrane associated proteins as well as protein P12 which is a non-structural viral protein necessary for the envelopment of the nucleocapsids (Sinclair et al. 1975; Emori et al. 1982; Johnson and Mindich 1994b). The late transcription phase is stopped by association of P8 with the procapsid to form the nucleocapsid (Olkkonen et al. 1991). These nucleocapsids are able to infect host cell spheroplasts *in vitro* (Ojala et al. 1990). *In vivo*, they become enveloped by a lipid membrane containing viral membrane proteins and virions are found in the cytosol fully detached from the host CM (Ellis and Schlegel 1974; Bamford et al. 1976). Protein P9 and P12 are essential in the morphogenesis of the viral membranes, since infection of the host cells with the mutants lacking either of these proteins resulted in the formation of non-enveloped nucleocapsids (Mindich et al. 1976). The other membrane proteins, P6, P10, P11 and P13, as well as the spike protein P3 are not necessary for membrane assembly. However, to produce infectious particles, the viral membrane must contain P6 to which the spike proteins are attached (Stitt and Mindich 1983b). The progeny phi6 virions (~150-200 particles per infected cell) are released by the cell lysis which is dependent on the action of the lytic protein P5 and membrane protein P10 (Mindich and Lehman 1979; Johnson and Mindich 1994a).

The nucleocapsid envelopment is temperature sensitive, since infectious particles were not produced when infected cells were shifted from 25 to 31 ºC before the time of membrane morphogenesis (Sands et al. 1974). It was shown that the temperature sensitivity for release of the virions is a protein-related effect, but was not due to impaired lysis.

Lipid envelope. The first evidence that suggested the presence of lipids in phi6 was the 1.27 g/cm^3 buoyant density of the virions in CsCl and sensitivity to detergents, organic solvents and phospholipase A (Vidaver et al. 1973). In electron micrographs phi6 particles seemed to have a lipid membrane as the outermost layer (Vidaver et al. 1973; Ellis and Schlegel 1974). Further analyses showed that virions contained approximately 25% lipids, of which PE constituted 35%, PG - 57%, and CL – 8% CL (Sands 1973). The PL composition

of phi6 was different from that of the host, but the FA compositions of the two were identical (Sands 1973; Vidaver et al. 1973). Before membrane assembly, the viral membrane proteins are associated with the host CM (Mindich et al. 1982), suggesting that the lipids of the virion are derived from the CM.

The exact mechanism of the phi6 nucleocapsid envelopment by the lipid membrane is not clear, but the observation that mature phi6 virions are localized centrally within the host cells indicates that it does not involve the typical budding mechanism, which is used by enveloped animal viruses (Garoff et al. 1998). A possible intermediate structure in the assembly pathway of phi6 membrane has been observed in the cells infected with the wild-type phi6 (Stitt and Mindich 1983a). The particles obtained had the density of 1.18 g/ml and contained the major viral membrane protein P9 (therefore called P9-containing particle), several minor membrane proteins and PLs. The PG/PE ratio in this particle was higher than that of uninfected cells. After addition of unlabelled amino acids, the labeled membrane proteins were chased into the phage, suggesting that the P9-containing particle was a precursor for the virion. This idea was supported by the observation that P9-containing particles were not produced when P12 was defective. In addition, protease and phospholipase treatments of the P9-containing particle had the same effects as treatment of intact phi6 virions (Stitt and Mindich 1983a, b), suggesting that the membrane topology is the same as in the complete virion.

Stitt and Mindich (1983) proposed several alternative mechanisms for maturation of phi6 virions which would involve the observed P9-containing particle (**Figure 10**).

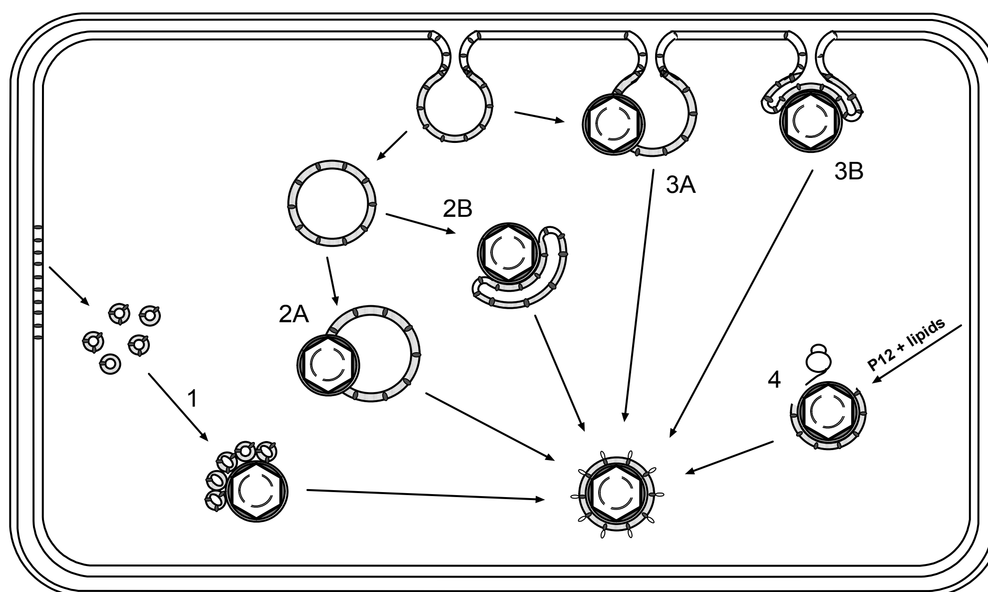

Figure 10. Models for assembly of membrane onto the nucleocapsids of phi6. See text for details. The picture is redrawn according to Stitt and Mindich (1983a).

The first mechanism (**Figure 10,** model **1**) was, proposed on the basis of observation that viral nucleocapsids could not be found in association with the host CM (Ellis and

Schlegel 1974) and that the synthesis of PG is increased in the late stages of infection (Sands and Lowlicht 1976). This mechanism involves the detachment of small PG-rich vesicles (micelles were proposed originally) containing viral membrane proteins from the inner leaflet of the host CM (site of increased PG synthesis), diffusion of those vesicles through the cytosol, attachment to the viral nucleocapsid and subsequent fusion of several of such vesicles to form the bilayer of the viral envelope (Sands and Lowlicht 1976). Protein P12 was suggested to aid the formation of these vesicles or their association with the nucleocapsids.

According to the second model (**2A** and **2B**), insertion of the viral membrane proteins into the host CM induces the formation of membrane patches that have PL and protein composition similar to those of the virus. Protein P12 could mediate the invagination of these patches and the detachment of free phage-sized vesicle from the host CM. The preformed nucleocapsid then either penetrates into this vesicle (model **2A**) or the vesicle wraps around the nucleocapsid (model **2B**). P12 could also mediate vesicle association with the nucleocapsid.

The third model (**3A** and **3B**) is similar to the second, but in this case the protuberant patch of the host CM rich in viral proteins and lipids is not released into the cytosol until the viral nucleocapsid either penetrates into it (model **3A**) or is wrapped by the membrane extrusion (model **3B**). P12 could mediate the pinching-off of the protuberant-like patches to release mature virions into the cytosol. Observation that in cells infected with mutants defective in P12 synthesis nucleocapsids appear to be trapped in "sacks" of host CM support models 3A and 3B as well as the pinching-off function for P12 (Bamford 1980). According to the model **4**, protein P12 may act as a PL transfer protein picking lipids from the host CM and delivering them onto the NC simultaneously with viral membrane proteins synthesized at the surface of the NC.

Each of these mechanisms envisage the formation of a vesicle which is similar to the P9-containing particle observed in phi6-infected cells (Stitt and Mindich 1983a). In models 1 and 4 the P9-containing particle would represent the incomplete viral membranes that have detached from the nucleocapsids upon lysis, suggesting that the incomplete precursors of the viral membrane could be formed only in the presence of nucleocapsids. It has been reported, however, that cells carrying plasmid directing the synthesis of P12 and infected with phi6 mutants defective in P8 (the last protein added to form the nucleocapsid) and P12 can still produce P9-containing particles (Mindich 1988). Procapsids lacking P8 were also produced in these cells. Furthermore, expression of proteins P9 and P12 in non-infected cells produce particles similar to those produced in phi6-infected cells (Johnson and Mindich 1994b). These latter observations indicate that the nucleocapsid is not necessary for the production of the precursor membrane, thus excluding models 1 and 4. Furthermore, models **2A** and **3A** predict that the outer leaflet of the precursor membrane and of mature phi6 virions is derived from the inner leaflet of the host CM (and vice versa for model **2B** and **3B**). Therefore the studies of transbilayer distribution in phi6 (and in the host CM) might help to rule out some of those remaining pathways.

2 AIMS OF THE PRESENT STUDY

The aim of this study was to determine detailed PL class and molecular species compositions in the membranes of bacteriophages PM2, PRD1, Bam35, and phi6 as well as of the membranes of their respective host. A further aim was to determine PL class and molecular species compositions of individual leaflets in bacteriophage membranes. To reach these goals, several tasks had to be performed:

a) To determine PL class as well as the molecular species compositions of bacteriophages PRD1, Bam35, phi6 and PM2.

b) To isolate the CM and OMs of Gram-negative bacteria *Salmonella enterica sv.* Typhimurium DS88, *Pseudomonas syringae* pv. phaseolicola HB10Y and *Pseudoalteromonas sp*. strain ER72M2.

c) To determine PL class and molecular species compositions of CM and OM of Gram-negative and CM of Gram-positive hosts of all studied bacteriophages.

d) To determine the transbilayer distribution of phospholipids (and molecular species) in bacteriophage membranes.

Comparison of the phospholipid compositions of viral and host membranes should allow evaluating whether selection of phospholipids takes place during virus assembly. Total PL compositions and PL transbilayer distribution data allow calculation of the PL compositions of individual membrane leaflets. Studies on the transbilayer PL distribution should tell whether membrane asymmetry may be responsible for PL selection to viral membranes. These data should also be useful when interpreting high-resolution structural data obtained for these bacteriophages.

3 EXPERIMENTAL PROCEDURES

3.1 Viruses and bacteria

In this study, bacteriophages PRD1, Bam35c, phi6 and PM2 and their respective hosts *Salmonella enterica sv.* Typhimurium DS88, *Bacillus thuringiensis* HER1410, *Pseudomonas syringae* pv. phaseolicola HB10Y and Pseudoalteromonas sp. strain ER72M2 were used. In addition, *Salmonella enterica* suppressor strain PSA was used to propagate PRD1 *sus1* mutant (deficient in genome packaging).

3.2 Production and purification of viruses (I, II, III)

The bacteria were grown in LB medium with aeration at 37°C (or 28°C) to stationary phase, after which they were diluted 1:10 with LB and incubated with aeration at the same temperature until they reached 2-5×10^8 CFU/ml density. Fresh agar stocks were added to the suspension to obtain a multiplicity of infection (MOI) of 10 and incubation was continued until lysis. The lysates were cleared from cell debris, and viruses were precipitated by polyethylene glycol (PEG) and NaCl and suspended in a small volume of buffer solution. The concentrated virus was then purified by rate zonal (5-20% sucrose gradient) and equilibrium (20-70% sucrose gradient or CsCl gradient) centrifugations to obtain ultra-pure virus. To remove flagellar contamination from Bam35 preparations, ammonium sulfate precipitation was done before equilibrium centrifugation. The virus-containing zone from the equilibrium gradient was collected, diluted 3 times with the buffer solution, and virions were collected either by differential centrifugation (PRD1, Bam35, phi6 and PM2) or by filtration through concentrator filters (phi6). The pellet was then gently suspended in the buffer used for the specific experiments. The virus preparation was used immediately (in transbilayer distribution studies) or stored in -20C until analyzed (in lipid composition studies).

3.3 Preparation of the membranes from bacteria (I, II, III)

The bacteria diluted 1:10 were grown in LB medium with aeration at 37°C (or 28°C) to reach the cell density used for virus infection. The cells were harvested, the pellet washed twice with 10 mM Tris-HCl, pH 7.4 and suspended in 4 ml of the same buffer.

For the disruption of Gram-positive *Bacillus thuringiensis* HER1410 cells, lysozyme was added to a final concentration of 5 mg/ml and the mixture was sonicated twice for 10 seconds on ice (Filgueiras and Op den Kamp 1980; Lacombe and Lubochinsky 1988) with maximum intensity. The mixture was then incubated for 20 minutes at 22°C to allow digestion of the peptidoglycan layer after which the lipids were extracted.

The OM and CM membranes of *Salmonella enterica* DS88 were obtained using previously described procedures (Osborn et al. 1972b; de Maagd and Lugtenberg 1986) except that a flotation gradient was utilized. Briefly, the cell suspension was supplemented with lysozyme, DNase and RNase (0.1 mg/ml each) and passaged twice through a cold French Pressure Cell (500–850 atm) to disrupt the cells. After the removal of unbroken cells and aggregated cell debris by centrifugation lysozyme, DNase and RNase were again added to a final concentration 0.2 mg/ml each, and the mixture was incubated at 22°C for 20 minutes and then diluted (1:2) with a buffer containing ethylene diamine tetraacetic acid (EDTA). Following the addition of KCl (final concentration 0.2 M), the total membrane fraction was collected by differential centrifugation. The pellet was rinsed once, suspended in 1ml of the same buffer, and solid sucrose was dissolved in the suspension to obtain the concentration of ~62% (w/w). Half a milliliter of this membrane suspension was layered onto a sucrose cushion (67%, w/w) in a Beckman SW 41 centrifuge tube and the sample was overlaid with discontinuous sucrose gradient (I, II). After separation of the OM and CM by flotation gradient centrifugation (35000 rpm, 72 hr, 4 °C), 0.5 ml fractions from one tube were collected, and their densities were determined based on the refractive index. Protein concentration, 2-keto-3-deoxyoctonate (KDO) content and light scattering were also determined for each fraction. The light-scattering zones from other tubes were collected, diluted 3-5 times, pelleted by centrifugation and suspended in a small volume of a buffer solution. The membranes were then analyzed immediately or stored at –20°C until lipid extraction.

The CM and OM of *Pseudomonas syringae* were separated as described above except that EDTA was not added at any step of the separation (Hancock and Nikaido 1978).

3.4 Preparation of total membranes during phi6 infection (II)

To evaluate whether phi6 affects the PL synthesis of *Pseudomonas syringae*, the HB10Y host was grown and phi6 infection was carried out as described above. Aliquots of the infected and uninfected (negative control) cell cultures were taken at various time points before and after infection. Cells, membranes of lysed cells and mature phi6 virions were collected by high-speed centrifugation, suspended in a buffer solution, and lipids were extracted immediately.

3.5 Lipid extraction and class distribution determination (I, II, III)

Lipids were extracted according to a standard protocol (Folch et al. 1957) and stored in chloroform/methanol (9:1, v/v) at -20°C. Thin-layer chromatography (TLC) was carried out on Silica Gel 60 plates (Merck) using chloroform/methanol/acetic acid (65:25:10, v/v, Kamio and Takahashi 1980) as solvent. The lipid bands were visualized with iodine vapor, scraped from the plate and quantified by the phosphate determination (Kahma et al. 1976).

3.6 Mass spectrometric analysis of phospholipids (I, II, III)

ESI-MS was used to identify and quantify the lipids. The identities of the lipids were determined based on three parameters: *i*) their m/z in the negative and positive ion mode *ii*) by head-group specific ESI-MS/MS scanning (PE species were identified by scanning for neutral loss of 141 in the positive ion mode (Kerwin et al. 1994; Brugger et al. 1997), while PG species were determined by scanning for precursors of 171 in the negative ion mode (Hsu and Turk 2001)), and *iii*) the identity of each PE and PG species was confirmed using ESI-MS/MS daughter ion scans (Hsu and Turk 2000). To determine the lipid composition by MS, aliquots of lipid extracts were spiked with internal standards consisting of di-14:1, di-20:1 and di-22:1 PE, di-14:1 and di-20:1 PG as well as tetra-14:0 and tetra-18:1 CL to correct spectra for the effects of acyl chain length on instrument response (Koivusalo et al. 2001). The MS analyses were then carried out essentially as described before (Koivusalo et al. 2001; Käkelä et al. 2003). Quantification of the molecular species compositions was accomplished either by ESI-MS (III) or by liquid chromatography ESI-MS (**I, II**).

3.7 Gas-liquid chromatographic analysis of fatty acid methyl esters (I, II, III)

Fatty acid methyl esters were obtained by transmethylating the lipid extracts in 1% H_2SO_4 in methanol for 90 minutes at 95°C, and analyzed by a gas-liquid chromatograph using DB-wax capillary column with both flame ionization (FID) and mass detection. The FAs were identified by comparing their retention times and mass spectra with those of authentic (Sigma) and natural standards. The FID responses were corrected according to Ackman (1991) to determine molar proportions of FAs within the sample.

3.8 Multivariate analyses of the lipid compositions (II)

To evaluate how different the viral and bacterial membranes are, the PL and FA compositional data were subjected to multivariate principal component analysis (PCA) using the SIRIUS 6.5 software package (Pattern Recognition Systems Ltd., Bergen, Norway, Kvalheim and Karstang 1987). In the PCA bi-plots the relative positions of the samples and variables were displayed on a plane defined the principal components PC 1 and PC 2 representing the largest and second largest variances among the samples and variables.

3.9 Determination of the transbilayer distribution of phospholipids (III)

PG in the outer leaflet was selectively oxidized with sodium meta-periodate at room temperature (RT) or on ice. At various time points aliquots of the reaction mixture were removed and the reaction was stopped by sodium thiosulfate followed by incubation on ice for 5 min. PE in the outer leaflet was selectively derivatized with 5 mM (final concentration)

TNBS at RT or on ice. The reaction was stopped by mixing aliquots of the reaction mixture with ice-cold quenching buffer (0.5 M glycylglycine, 0.1 M citric acid, pH 5.0) incubating on ice for 5min. The samples were then stored at −20 °C until extracted and analyzed.

The outer leaflet PLs of bacteriophage phi6 membrane were also selectively digested with bee venom phospholipase A_2 (PLA_2; 1–5 ng/100 nmol of viral PL) and incubated at RT. Aliquots were taken at intervals and the was reaction stopped by 5 mM EDTA (pH ~2), incubated on ice for at least 5 min and kept at −20 °C until extracted.

Parallel reactions were also performed while sonicating (tip sonicator, 1–2 min pulses; on ice) the reaction mixtures in order to determine the level of PL modification when both leaflets were accessible to the modifying reagents.

Lipids from the samples were extracted as described above, except that the mixture of internal standards was added to the final lower phase, followed by evaporation under nitrogen stream. Lipids were dissolved in 100–200 μl of chloroform/methanol/25%NH_3 (25:50:3; v/v) and analyzed immediately by ESI-MS operated at negative mode with essentially the same settings as described elsewhere (Hermansson et al. 2005). When necessary, specific scans for PE and PG were performed to selectively detect and quantify the molecular species.

3.10 Data analysis (I, II, III)

The peak lists of MS spectra were copied to Microsoft Excel and the PL molecular species were quantified using the LIMSA software tool developed in the laboratory (Koivusalo et al. 2001; Haimi et al. 2006).

3.11 Other methods (I, II, III)

The Coomassie brilliant blue method (Bradford 1976) was used to determine the protein concentration using bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 16% acrylamide) of bacterial membranes was carried out as described (Olkkonen and Bamford 1989) and thiobarbituric acid assay (Keleti and Lederer 1974) was used to determine 2-keto-3-deoxyoctonate (KDO) content. Light scattering of the gradient fractions was measured by a PDI 2020 detector (Precision Detectors, Inc.).

4 RESULTS AND DISCUSSION

4.1 Separation of CM and OM of Gram-negative bacteria

The total membranes of *Salmonella enterica* DS88, *Pseudoalteromonas sp.* strain ER72M2 and *Pseudomonas syringae* HB10Y were obtained by combining the procedures described previously (de Maagd and Lugtenberg, 1986; Osborn et al., 1972). The CM and OM were separated using a flotation gradient equilibrium centrifugation. The average densities of the fractions containing the two light-scattering bands (1.24 ± 0.02 and 1.18 ± 0.03 g/ml) were in agreement with previously published values for the OM and CM, respectively, of other Gram-negative enteric bacteria (Schnaitman 1970; Osborn et al. 1972a; Diedrich and Cota-Robles 1974; Ishinaga et al. 1979; Cronan et al. 1987). In all cases, the lower band was identified as the OM as it contained the major fraction of KDO, a marker for the OM (Keleti and Lederer 1974). Consistent with earlier studies on *E. coli* and *S. Typhimurium* (Kadner 1996; Nikaido 1996), the putative CM fraction had higher protein concentration than the OM fraction **(II, Figure 2)**. The third protein peak (in bottom fractions) represented the soluble proteins that where associated with the total membrane fraction. SDS-PAGE analysis showed negligible cross-contamination between the OM and the CM. These data indicate that flotation gradient centrifugation is an excellent method to obtain highly purified CM and OM fractions.

4.2 Phospholipid and fatty acid compositions

Before this study, the PL class compositions of the two bacteriophages used here, i.e., PM2 (Braunstein and Franklin 1971; Brewer and Goto 1983) and phi6 (Sands 1973), and a tectivirus PR4 (Davis et al. 1982) had been studied. The validity of the previous PM2 data was, however, in doubt, since the early structural model of this phage had been re-evaluated (Kivelä et al. 1999; Kivelä et al. 2002). To solve this issue and to evaluate the structural data on PM2 (Huiskonen et al. 2004, Abrescia et al., personal communication) and the two tectiviruses PRD1 (Martin et al. 2001; Bamford et al. 2002b; Abrescia et al. 2004; Cockburn et al. 2004) and Bam35 (Laurinmäki et al. 2005), the PL class and molecular species compositions of these phages (and phi6) were determined using a conventional TLC approach and quantitative ESI-MS. PL class compositions of the viral membranes were then compared with those of the purified host CM and OM. The results (articles **I-III**) show that the viral membranes contain relatively more PG and less PE as compared with the host CM and OM (see below).

4.2.1 PRD1

In agreement with previously published results (Osborn et al. 1972a), the CM of *Salmonella enterica* pv. typhimurium DS88 contained slightly less PE and more PG than the OM, and the PG to CL ratio was ~3 in CM, but only ~1 in OM (**I, Figure 1**). In PRD1, PG was enriched and PE depleted as compared to the host membranes (**I, Figure 1**). The PL class composition of PRD1 was quite similar to those of related bacteriophages, i.e., PR4 grown in *E. coli* strain TD6 (Davis et al. 1982) and PR5 propagated in *E.coli* K12 SA1306 (Wong and Bryan 1978). Contrary to other earlier studies (Sands and Cadden 1975; Sands 1976), PS was not detected. The reason for presence of PS in PR4 and its host *E.coli* CR34 in those studies is not known, but might possibly be due to impaired decarboxylation of PS (the precursor of PE).

The analysis of phospholipids was extended by studying PL molecular species compositions in bacteriophage and host membranes. Fragmentation analysis of each molecular species allowed identification of these FA residues (**I, II**). Quantitative analysis of molecular species compositions was achieved by including multiple internal standards (Koivusalo et al. 2001; Käkelä et al. 2003). The membrane of PRD1 contained the same PL molecular species as its host, and in similar proportions. The major PE and PG species in the viral and host membranes were 32:1 (16:0/16:1, i.e., 16:0 and 16:1 acyl chains), 34:1 (16:0/18:1), 34:2 (16:1/18:1) and 36:2 (18:1/18:1) (**I, Figure 2**). There were no molecular species present only in the virus or in the host, and only small differences in molecular species compositions between the viral and host membranes were observed. The similar molecular species compositions of the viral and host membranes suggested that the FA residues are far less important for the selection of PG to the membrane of PRD1 than the headgroup.

To back-up the fragmentation analysis of PL molecular species and to obtain additional information on the FA residues (e.g., position of the double bond, branching, etc.), we analyzed the total FA compositions of PRD1 membrane and the membranes of its host by gas-liquid chromatography (**I**). The major FAs in these membranes were 16:0, c9-16:1 and c11-18:1 (**I, Figure 3**), which are typical for enteric Gram-negative bacteria such as *Escherichia coli* (Marr and Ingraham 1962). The total FA composition of PRD1 was similar to that of PR4 determined previously (Davis et al. 1982) (**I, Table 1**).

4.2.2 Bam35

The CM of *Bacillus thuringiensis* HER1410, the Gram-positive host of Bam35, contained more PG (~30mol %) and less PE (~58mol %) than the CMs of Gram-negative bacteria. The membrane of Bam35 was further PG-enriched and PE-depleted as compared with its host (**I, Figure 4**). The PL class composition of Bam35 was very different from that of PRD1, although the overall architecture and the capsid structures of these two tectiviruses

are surprisingly similar. Different PL compositions of these viruses are probably necessary to obtain membranes with different curvature profiles that were observed in these phages (Laurinmäki et al. 2005). It is also possible that the PL class composition in Bam35 is so adjusted to allow optimal lipid and protein packing. A similar phenomenon has been observed in the Gram-positive FA auxotroph *Clostridium butyricum*. When given unsaturated FAs, it adjusts its PL composition towards bilayer forming lipids in order to counter-balance the tension invoked by newly synthesized "unnatural" unsaturated PEs (Goldfine et al. 1987).

Most PE and PG molecular species in Bam35 were either saturated or monounsaturated. Saturated molecular species comprised ~45% of PE and ~65% of PG. Only di-unsaturated molecular species of CL (equal to 2 monounsaturated PG molecules) were detected (**I, Figure 5**). The molecular species composition of Bam35 was quite similar to that of its host, but monounsaturated species tended to be slightly enriched in the membrane of the phage.

Most of FAs in *Bacillus thuringiensis* HER1410 and in Bam35 were branched (*iso* or *anteiso*) which is a common feature in Bacilli (Kaneda 1977). The most abundant fatty acid in Bam35 and HER1410 was *iso*-branched 15:0, the other species being much less prominent.

4.2.3 PM2

In agreement with previous data (Braunstein and Franklin 1971; Brewer and Goto 1983), we found that PM2 contains ~34% PE and ~66% PG (**III, supplementary material**). The CM of the host *Pseudoalteromonas sp.* strain ER72M2 contained more PE (~75mol %) and less PG (~25mol %) than the membrane of PM2. Similar values were obtained previously for the CM of another host of PM2, i.e. *Pseudoalteromonas espejiana* BAL31 (Diedrich and Cota-Robles 1974). Notably, the OM of the *Pseudoalteromonas sp.* strain ER72M2 was found almost identical to the CM (unpublished data). This is unusual, because the OM of most Gram-negative bacteria contain more PE and less PG than the CM (Osborn et al. 1972a; Ishinaga et al. 1979). Similar compositions of CM and OM are unlikely to be due to poor separation of the membranes, as most of the KDO was found in the OM fraction.

Molecular species analysis of PM2 and its host membranes showed that the PE and PG molecular species with longer acyl chains were slightly more abundant in the viral membrane than in the host CM, while the opposite was true for molecular species with shorter acyl chains (**Figure 11**), suggesting that the transmembrane helices spanning in PM2 might be slightly longer than those in the host membranes.

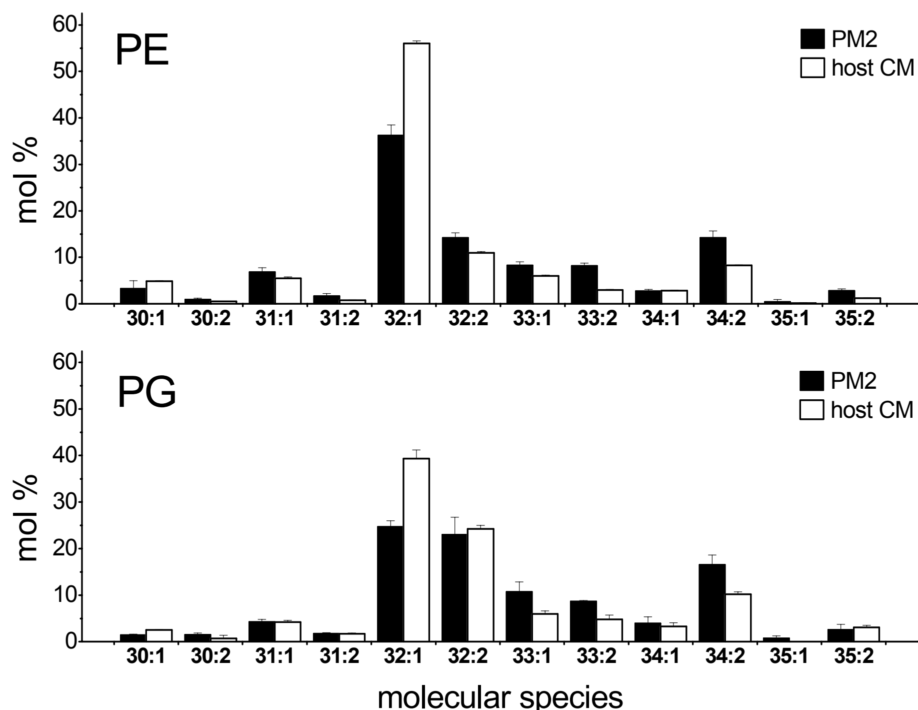

Figure 11. PE and PG molecular species compositions of bacteriophage PM2 and CM of its host *Pseudoalteromonas sp.* strain ER72M2. Compositions are expressed as mol % within each class. Bars and error bars represent averages and standard deviations, respectively (n=5 for PM2 and n=3 for host CM).

In addition to PE and PG that are the only phospholipids detectable by TLC, bacteriophage PM2 and its host contain trace amounts of PA and acyl-PG (e.g., 14:0, 16:1, 14:1-acyl-PG, di-16:1, 14:1-acyl-PG and 16:0, 16:1, 16:1-acyl-PG) detected by ESI-MS. Acyl-PG has previously been identified as a minor phospholipid of *Pseudoalteromonas espejiana* BAL-31 (Tsukagoshi et al. 1976a) and *Salmonella enterica* pv. typhimurium (Hsu et al. 2004).

4.2.4 Phi6 and other cystoviruses

The membrane of the enveloped bacteriophage phi6 was also enriched in PG (~38 mol %) as compared with the CM (~16 mol %) and OM (~10 mol %) of the HB10Y host (**II, Figure 3**). These results differ from those of a previous study, where it was found that PG comprised ~57% PG and PE ~35% of the total PLs of phi6 (Sands 1973). The reason for this discrepancy is not clear, but production of virus in post-exponentially growing cells (as indicated by the high PG + CL content) in that study could be one explanation. Alternatively, the extraction procedures used could have caused some degradation of lipids.

The enrichment of PG in phi6 in a previous study was attributed to increased synthesis of PG upon viral infection (Sands and Lowlicht 1976). We tested this possibility by monitoring the PL composition (or PG/PE ratio) of total membranes (i.e., intact cells, debris of cellular membranes and mature phi6 virions) in infected versus uninfected cultures during and after phage production. If the synthesis of PG or degradation of PE would be increased in infected cultures, the PG/PE ratio should also rise, or remain constant if the PL metabolism is unaffected. The results showed that during the maturation of phi6 virions (i.e., 0 – 100 min after infection) the PG/PE ratio in the total membranes was relatively constant (**II, Figure 4**), thus excluding the possibility that phospholipid metabolism is responsible for the increased level of PG in the viral membranes. Only at the end of lysis the PG/PE ratio increased which is probably due to the activation of PE-specific host OM-associated phospholipases (Cronan and Vagelos 1972) as previously suggested for PR4 (Muller and Cronan 1983) and PM2 (Diedrich and Cota-Robles 1976).

PL class compositions of two other cystoviruses, phi8 and phi13, grown on *Pseudomonas syringae* strain LM2489 were also studied. Despite different membrane protein compositions of phi6 and phi8 (Hoogstraten et al. 2000), the PL composition of phi8 was very similar to that determined for phi6 (unpublished data, not shown). Phi13, on the other hand, contained more PG and less PE than phi6 (**Figure 12**). Although the PL composition of the LM2489 strain has not been determined, it is reasonable to propose that it is similar to that of *Pseudomonas syringae* HB10Y (**II**), the host of phi6.

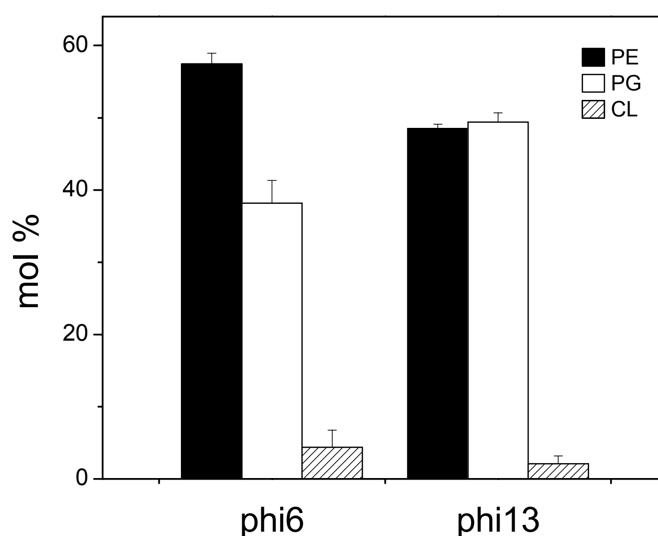

Figure 12. Phospholipid class compositions (expressed as mol % of the total PLs) of cystoviruses phi6 and phi13. Values for phospholipid proportions in phi6 membrane were taken from article **II**. Bars and error bars represent averages and standard deviations, respectively (n=7 for phi6 and n=3 for phi13).

As with the other bacteriophages studied, the molecular species composition of phi6 was quite similar to those of the CM and OM of *Pseudomonas syringae* host. However, some statistically significant differences were observed. For instance, phi6 contained relatively more 34:1 and 34:2 and less 32:1 PE and PG species than OM and (not in all cases) CM of its host (**II, Figure 5**).

In agreement with previously published data (Vidaver et al. 1973), 16:0, c9-16:1 and c11-18:1 fatty acids comprised more than 90 mol % of total FAs in phi6 and its host membranes (**II, Figure 6**). Statistical analysis showed that phi6 contained significantly less 16:0 and more c11-18:1 than the host membranes, while the relative amounts of other FAs were similar in all three membranes.

It is quite difficult to compare the PL compositions of viral membranes with those of the host CM and OM just by looking at the proportions of individual FAs or molecular species, especially when those proportions seem quite similar. To get a more objective comparison, we performed PCA with the molecular species data of bacteriophage phi6 and CM and OM of its host. It is evident **(II, Figure 7)** that, based on the major PE and PG molecular species, the composition of the host OM is different from that of the CM. The phi6 membrane composition is more similar to the CM, thus indicating that the host CM is likely to be the origin of phi6 phospholipids.

4.3 Transbilayer phospholipid class distribution in bacteriophage membranes.

To determine the PL composition of the individual membrane leaflets of phages, the transbilayer distribution of PL classes and molecular species were studied (**III**). This was achieved by selectively modifying phospholipids located in the outer membrane leaflet. In general, this can be accomplished by using reagents that cannot penetrate through the membrane bilayer (or the rate of penetration is much slower than the rate of lipid modification). In this study, sodium periodate was used to oxidize the non-acylated glycerol moiety of the PG (Lentz et al. 1980; Huijbregts et al. 1997), and TNBS to covalently bind to the amino group of PE molecules (Bishop et al. 1977; Rothman and Kennedy 1977; Brewer and Goto 1983) located in the outer leaflet. Since both of these reagents can penetrate through the bilayer to some degree (Hubbard and Cohn 1976; Huijbregts et al. 1997), the time-courses of reactions were determined to reliably distinguish the rapidly (outer) and slowly (inner) reacting PL pools. By extrapolation of a linear fit to the data representing the slower phase of the reaction (Zumdahl 1997) to zero time the fraction of the particular PL in the outer leaflet can be determined.

4.3.1 PRD1

The time-course curves of PG oxidation in bacteriophage PRD1 showed that ~56% of PG was localized in the outer leaflet (**III, Figure 1, panel A**). The pH (pH 7.4 vs. 8.5) or the

concentration of sodium periodate did not affect the size of this rapidly reacting pool. When the membrane was disrupted by sonication, PG reacted rapidly in a one-phase fashion showing that the slower phase was not due to the consumption of the reagent and that nearly all PG could be oxidized in the conditions where both leaflets were accessible to the reagent. To test whether DNA affects the transbilayer PL distribution in wt PRD1, parallel experiments were carried out with the *sus1* mutant of PRD1, which does not contain DNA due to the lack of the packaging ATPase P9. At room temperature, the oxidation of PG in *sus1* particles was very rapid and complete (**III, Figure 1, panel B**), indicating that periodate can penetrate the bilayer, most likely through the open portal vertexes (Strömsten et al. 2003). However, upon incubation of the reaction mixture on ice, the two pools could be readily distinguished, and ~52% PG was found in the outer leaflet pool (**III, Figure 1, panel B**), thus indicating that DNA does not influence the transbilayer distribution of PG in the membrane of PRD1.

Approximately 36% of PE could be assigned to the outer leaflet of the PRD1 membrane by incubation with TNBS at RT (**III, Figure 1, panel C**), consistent with a previous study on bacteriophage PR4 (a close relative of PRD1) in which less than half of PE could be modified with the non-permeable reagent, isethionyl acetimidate (Davis and Cronan 1985). The transbilayer distribution of PE in the *sus1* mutant was identical to that in *wt* particles (**III, Figure 1, panel D**), thus indicating that the distribution of PE neither is affected by DNA.

The structure of PRD1 has been recently solved by X-ray crystallography (Abrescia et al. 2004; Cockburn et al. 2004). Both leaflets of the viral membrane were visible in the electron density map, and the outer leaflet of PRD1 membrane contained more electrons than the inner one. This led the authors to suggest that the membrane of PRD1 is asymmetric with all PG and CL molecules residing in the outer leaflet and most of the PE molecules in the inner one (Cockburn et al. 2004). In our study, however, only 55% of PG was found in the outer leaflet [distribution of CL (~6 mol% of total PL in PRD1) was not determined in our study]. One possible reason for this discrepancy could be that the counter-ions (e.g., Na^+) and solvating water molecules contributing to the total electron density could not be resolved in the X-ray study and their exact number is not known. Another possible reason could be that the presence of membrane proteins, which are abundant in PRD1 (Davis et al. 1982; Grahn et al. 2006), was not taken into account when modeling the electron density data (Cockburn et al. 2004). Notably, we found that the number of lipid molecules in the inner and outer leaflets of PRD1 membrane is similar (**III, Figure 5**), despite the larger surface area of the latter. This suggests that a large area of the outer leaflet is taken up by proteins that were not resolved in the X-ray study. At least part of this area could be also filled by the major capsid protein P3, as it was shown that the N-termini of this protein are in contact with the outer leaflet of PRD1 membrane (Abrescia et al. 2004). This P3-membrane interaction is supported by the ability of the major capsid protein of the closely related bacteriophage PR4 to be cross-linked to PG molecules (Davis and Cronan 1985).

By plotting the modification of individual molecular species vs. time, their transbilayer distribution in PRD1 membrane could be determined. The transbilayer distribution of different molecular species within the same class was very similar in most cases (**III, Supplementary Figure 2**), except in few cases, where significant differences were detected. For instance, the 31:1, 32:2 and 33:2 PE species as well as the 30:1, 31:1, 32:2 and 33:2 PG species were more enriched in the inner leaflet as compared to the total PG or PE class, respectively. On the other hand, the longer and more saturated PE species, such as 32:0, 35:1, 35:2 and 36:2, were slightly more abundant in the outer leaflet than the PE as a class (see below for possible explanation).

4.3.2 Bam35

In this bacteriophage the majority of both PG (~57%) and PE (~54%) was found in the outer leaflet (**III, Figure 3**). Phospholipid distribution in empty (containing no DNA) particles of Bam35 was essentially the same as in intact virions, indicating that also in this phage the presence of DNA does not affect the lipid distribution. The transbilayer distribution of PE in Bam35 was different from that in the PRD1 membrane, possibly due to different properties of the molecular species present (see below).

The distribution of the individual molecular species was similar to that of the respective PL class. Nevertheless, most saturated PG species tended to be more enriched in the outer leaflet than unsaturated PGs with the same total acyl chain length (29:0 vs. 29:1, 30:0 vs. 30:1, etc.), except that the shortest saturated PG specie, 27:0, was more abundant in the inner leaflet than PG on the average (**III, supplementary material**). This tendency of the saturated species to prefer the outer leaflet (and vice versa for unsaturated species) indicates that the acyl chains also play a role in the asymmetric distribution of PL molecules in the viral membranes. Membrane areas coinciding with the vertices of icosahedral bacteriophages (PRD1, Bam35 and PM2) are the most highly curved areas, where the preferential transbilayer distribution of molecular species should be most evident. It could therefore be that some molecular species (and classes) tend to segregate laterally to these highly curved areas of the membrane at the vertices. For instance, saturated PG molecular species, due to their inverted cone-shape should prefer the outer leaflet of the vertices, whereas PE molecules with short/unsaturated chains, because of pronounced cone shape, are likely to prefer the inner leaflet of the vertices.

4.3.3 PM2

The membrane of bacteriophage PM2 was permeable to sodium periodate and TNBS at room temperature, thus making it difficult to determine the phases of labeling. This was overcome by performing the modification at lower temperature. Most of PG (~59 %) and ~40% of PE was found in the outer leaflet (**III, Figure 2**). Previously, membrane asymmetry in bacteriophage PM2 was addressed in two independent studies. In the first study (Schäfer et

al. 1974), the distributions of PG and PE were qualitatively similar as those determined here, but the absolute values were significantly different. The reason for this is not clear, since the kinetics of PL labeling by sulfanilic acid diazonium salt used in that study was not analyzed. In the other study (Brewer and Goto 1983), the transbilayer distribution of PE in PM2-specific membrane vesicles produced in host cells upon infection with the temperature-sensitive mutant of PM2 defective in the synthesis of the major coat protein was determined. In contrast to the present study, more than half of PE was found in the outer leaflet (Brewer and Goto 1983). A possible explanation is that the PL distributions in intact PM2 virions and in PM2 membrane vesicles are different.

The transbilayer distributions of PG molecular species in PM2 were different from those of PE molecular species. PG species containing shorter acyl chains (30:1, 30:2, 31:1, 31:2, and 32:1) were more enriched in the inner leaflet than PG as a class, whereas 32:2 and species with longer acyl chains (33:1, 33:2, 34:1, 34:2, and 35:2) tended to reside more in the outer one (**III, Supplemetary Figure 2**). PE molecular species, on the other hand, tended to distribute according to the degree of unsaturation, i.e., monounsaturated species preferred the outer leaflet, and di-unsaturated the inner one, consistent with their preferential locations according to the effective shape of the molecules. A possible explanation for this finding is that the distribution of PG in the PM2 membrane is determined (at least to some extent) by factors other than matching the lipid shape with the high curvature of the membrane. As negatively charged lipids tend to be at the boundary of the integral membrane proteins (Marsh 1990), it is possible that the distribution of PG molecular species could be influenced by electrostatic lipid-protein interactions. Specific molecular species could also be necessary to ensure optimal insertion of the integral membrane proteins into the viral membrane bilayer.

4.3.4 Phi6

Modification of phospholipids by chemical reagents indicated that in the membrane of this bacteriophage ~52% of PG and ~35% of PE resided in the outer leaflet (**III, Figure 4, panels A and B**). To confirm this result, the outer leaflet PLs were hydrolyzed by phospholipase A_2 (PLA_2). Essentially the same fractions of PG and PE were rapidly modified as with the chemical reagents (**III, Figure 4, panels C and D**), validating that these fractions are located in the outer leaflet. Removal of spike proteins of phi6 by butylated hydroxytoluene or by proteinase digestion did not affect the size of the fraction accessible to the phospholipase, indicating that the outer leaflet PLs were accessible to the enzyme in the intact virions as well. With PLA_2 the transbilayer distribution of CL could also be determined, and ~50% was found in the outer leaflet.

One of the missing pieces in the puzzle of bacteriophage phi6 is the morphogenesis of its lipid envelope, and this study on transbilayer PL distribution in phi6 might help to understand this mechanism. It has been shown that vesicular stomatitis virus and Semliki

Forest virus grown in baby hamster kidney (BHK-21) cells have a very similar membrane PL asymmetries (Patzer et al. 1978; van Meer et al. 1981; Allan and Quinn 1989), which are qualitatively similar to that of the host PM. It is therefore believed that PL asymmetry of the host cell PM is maintained in the viral membranes. In this study, the three bacteriophages infecting Gram-negative hosts (PRD1, PM2 and phi6) were found to have qualitatively similar PL transbilayer asymmetries, suggesting that the topology of the membrane leaflets in phi6 may be the same as in PRD1 and PM2 in which the outer leaflet PLs of the phage are derived from the inner leaflet of the host CM (Huiskonen et al. 2004; Grahn et al. 2006). Based on the above assumption, only models **2A** and **3A** (**Figure 3**) could be considered for the assembly of phi6 membrane, where the outer leaflet of the virus is derived from the inner leaflet of host CM. Furthermore, there is evidence from the electron-microscopic studies of cells infected with $P12^-$ mutant of phi6, suggesting that the phi6 nucleocapsid enters the periplasmic space (as in model **3A**) and buds back to the cell cytosol (Bamford 1980). It was shown that upon cell lysis the nucleocapsids remain associated with the protuberant structures of the host CM. This observation also suggested that P12 is necessary for the pinching-off of the enveloped nucleocapsid into the cytosol. To unambiguously confirm the validity of this (or any other) model for phi6 membrane assembly, one would need to determine transbilayer distributions of PLs in the CMs of the hosts of these bacteriophages; a task which has never been fully accomplished due to difficulties in obtaining intact and stable protoplasts of Gram-negative bacteria.

The remaining question in this model would be how the viral nucleocapsid enters the periplasmic space. Is it mediated by protein P8, which has been suggested to be involved in the nucleocapsid entry and its disassembly (Romantschuk et al. 1988; Poranen et al. 1999; Tuma et al. 1999). Or is there a large pore formed by the holin-like proteins in the host CM through which the nucleocapsid could reach the periplasmic space and bud back into the cytoplasm through the host CM?

4.4 Phospholipid composition of individual leaflets of bacteriophage membranes

Determination of the PL compositions and their transbilayer distribution allowed us to calculate the PL compositions of the individual leaflets in bacteriophage membranes (**Table 1**). One observation was that the fractions of total PLs in the inner and outer leaflets of bacteriophage membranes were quite similar. In some phages, the outer leaflet contained less PL than the inner one (assuming that the distribution of CL was symmetric). Such a distribution of total PLs was unexpected since the surface area of the outer leaflets in these bacteriophages is considerably larger than that of the inner one. Even when considering the larger size of fully hydrated PG headgroup [59Å^2 as compared with 49Å^2 of PE headgroup (McIntosh and Simon 1986; Garidel and Blume 2005)], only a small fraction of the „unfilled“ area could be accounted for. The possibility that lipids in the outer leaflet are packed less densely than in the inner leaflet is unlikely, since transbilayer movement of lipids

would probably even out such difference in the lateral pressure between the leaflets. More probable explanation is that proteins occupy larger area fraction in the outer leaflet than in the inner one. As mentioned above, there is evidence suggesting that in PRD1 (and PR4) a large part of the outer leaflet could be taken up by major coat proteins (Davis and Cronan 1985; Abrescia et al. 2004), or alternatively it can be occupied by peripheral domains of integral membrane proteins.

Table 1. *Phospholipid compositions*[a] *of the inner and the outer membrane leaflets of bacteriophages*

Phage	Inner leaflet				Outer leaflet			
	Total PL[b]	PE	PG	CL[c]	Total PL	PE	PG	CL
PM2	48.1±4.8	46.1±7.5	53.9±6.6	n.d.[d]	51.9±4.8	29.4±5.3	70.6±7.5	n.d.
PRD1	53.1±2.8	60.2±4.5	34.8±3.2	5.0±0.4	46.9±2.7	40.2±4.8	53.8±4.3	6.0±0.5
Bam35	44.8±4.3	30.8±7.4	50.8±5.5	18.4±2.1	55.2±5.1	29.5±7.1	55.5±5.7	15.0±1.7
phi6	57.4±3.0	64.9±3.3	31.4±3.3	3.8±2.1	42.6±2.8	47.4±4.0	47.4±4.4	5.2±2.8

[a] Compositions expressed as mol% of the total PLs in a given leaflet; ±SD.
[b] The proportions of total PLs are expressed as mol% of the total PLs in the whole membrane assuming that CL in PRD1 and Bam35 were equally distributed.
[c] The proportions of CL in the leaflets of PRD1 and Bam35 were calculated assuming that it is equally distributed between the leaflets.
[d] Not detected.

Analysis of the PL compositions of individual leaflets also revealed that PG constituted ≥47 mol % in the outer leaflet in all phages as well as in the inner leaflet of PM2 and Bam35 (**Table 1**). According to the superlattice model (Somerharju et al. 1999), proportions of negatively charged lipids (in this case PG + CL) higher than 33.3 mol% are energetically unfavorable due to mutual electrostatic repulsion. However, some negatively charged PG molecules probably interact with positively charged amino acid residues of proteins, which neutralizes their charge. Alternatively, the negatively charged lipid molecules could be neutralized by counter-cations (such as Na^+, K^+, Ca^{2+} etc.) as recently suggested in a molecular simulation study (Zhao et al. 2007). This would also be consistent with the observation that bacteriophage PM2, replicating in a marine bacterium that grows in a high-salt medium (Kivelä et al. 1999), had the highest PG content.

Molecular species compositions of the individual leaflets were not calculated. However, it is reasonable to assume that they would be similar to those of the bulk compositions of phage membranes (**I-III**), since the transbilayer distributions of individual molecular species were very similar to those of their respective class (**III**).

4.5 Factors influencing the phospholipid composition of bacteriophage membranes

The observation that the membranes of all (here and previously) studied bacteriophages contain relatively more PG and less PE than the membranes from which they are derived, suggested that there is a common factor which favors selection of PG to (or exclusion of PE from) the viral membranes. We initially thought that physico-chemical properties of PL molecules together with the asymmetric distribution of PG and PE in the highly curved viral membranes could account for the enrichment of PG (see section **1.4.4**).

Preferential localization of PG in the outer leaflet and PE in the inner one (except Bam35; see below) is consistent with the effective lipid shape theory (Israelachvili 1973; Israelachvili et al. 1980) which envisions that PG and PE would tend to localize in the outer and inner leaflets, respectively, of highly curved membranes based on their shape and charge (Cullis et al. 1986; de Kruijff 1997). In Bam35, PE is preferentially located in the outer leaflet, which seems inconsistent with this shape concept. However, molecular species analysis of Bam35 lipids showed that all PE species are either saturated or monounsaturated. Such PE species resemble more a cylinder in shape (Israelachvili et al. 1980), and therefore should have no preference for the inner leaflet of viral membranes, unlike more unsaturated PE species.

This initial "hypothesis" was, however, ruled out by the finding that the number of lipids in both leaflets of the phage membranes was quite similar. Therefore, the observed PG enrichment in the outer leaflet is not alone sufficient to account for the overall enrichment of PG in the viral membrane because PE is enriched in the inner one to approximately the same extent. Moreover, analyses of PL compositions of individual membrane leaflets (**Table 1**) showed that also the inner leaflet of the viral membranes contained relatively more PG than the average PG content of the host CM. This enrichment of PG in the inner leaflet could be accomplished by two alternative mechanisms. First, the inner leaflet of the viral membrane might be derived from the pre-existing PG-rich domains of the host CM. It has been suggested that PG-rich domains segregate in the membranes of Gram-negative and Gram-positive bacteria (Vanounou et al. 2003). However, a recent molecular simulation study indicated that PG prefers to interact with PE rather than with itself (Murzyn et al. 2005) thus suggesting that PG-rich domains may not be formed. A second possibility is that viral membrane proteins, inserted into the host CM, attract PG molecules thus enriching PG via positively charged amino acids in the vicinity of their transmembrane helixes. Since the topologies of most membrane proteins in bacteriophages are not known at present, it is impossible to estimate whether these interactions would enrich PG in the inner or the outer leaflet (or both). Notably, the observation, by X-ray crystallographic analysis of bacteriophage PM2 (Abrescia et al., personal communication), that the positive amino acid residues in the major integral membrane protein P3 reside at the inner surface of the

membrane, suggests that such a mechanism for PG enrichment in the inner leaflet is feasible at least in some cases.

4.6 Significance of the bacteriophage phospholipid compositions

Phospholipid compositions of bacteriophage membranes are established at the time of membrane assembly at the host CM, and remain constant throughout the phage life-cycle. PL compositions of individual leaflets seem to be determined by 1) intrinsic properties of phospholipid molecules, i.e., by their effective shapes matching the curvature of the viral membrane leaflets and 2) the (specific) interactions of lipids with viral proteins. Therefore, these two factors might be necessary for the optimal packing of lipids and viral proteins within the viral membrane. In addition to the optimal packing, different PL compositions of the leaflets might also be necessary for the interaction of the phage membrane with the DNA and/or the viral coat, or even for the proper linking of the viral membrane to the spike complex (e.g., in PRD1), thus stabilizing the virion, which is most critical after their release from the host.

On the other hand, upon infection, the PL compositions of the phage should support (or even promote) the interaction of the viral membrane with the host cell. For instance, high amounts of PG and PE in the outer and inner leaflets of the viral membrane, respectively, might be necessary for the formation of the tubular membrane extension in PRD1 and Bam35 through which the DNA is delivered to the cytosol. Different transbilayer distributions of PLs in bacteriophages PM2 and phi6 might also be required for the fusion of the viral membrane with the host OM.

5 CONCLUDING REMARKS

This study shows that the assembly of bacteriophage membranes from the host CM is a selective process in respect to proteins, but to phospholipids as well. This selectivity is most evident in the case of PG enrichment (and PE depletion) in the viral membranes as compared with the host membranes. PL acyl chains appear to be less important than the headgroup for selection of lipid for the phage membrane, since both PG and PE molecular species compositions of the viral membranes were very similar to those of the host.

The reasons for selective incorporation of host phospholipids to bacteriophage membranes are not clear, but it seems likely that the effective shape and charge on the phospholipid molecule are the main factors. As suggested by asymmetric phospholipid distribution, the selective incorporation of host lipid to the phages membrane could partially derive from packing constraints in the highly curved phage membranes. PG in phage membranes could be partially enriched due to interaction with viral proteins which seem to occupy larger area in the outer membrane leaflet than in the inner one. Taken together, the PL composition of bacteriophage membranes (and individual leaflets) most likely represents the optimal protein and lipid packing, and is also determined by the properties of lipids available in the host.

Differences in the effective shape and charge between PG and PE are also likely to contribute to the asymmetric transbilayer distribution of these phospholipids in the phage membranes. PG as a cylindrical lipid fits better in the positively curved outer leaflet, while the cone-shape of PE is more compatible with the negatively curved inner layer. The phospholipid acyl chains, on the other hand, seem to have a rather minor effect on the transbilayer phospholipid distribution, possibly because the physical properties of different molecular species within a class are quite similar.

Analysis of individual leaflets also showed that both sides of the membrane are different in terms of PL compositions. This is consistent with the observation that both leaflets of viral membranes have different electron densities when determined by X-ray crystallography. However, as there are also other components (proteins, ions) in the viral membrane, the bulk electron density cannot be used to quantitatively determine the PL transbilayer distribution. Therefore the information collected in the present study should be useful when interpreting structural data obtained for the membranes of these bacteriophages.

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