Synthesis and protein curing abilities of membrane glycolipids

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Tillägnad mamma och pappa
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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>bisPDPC</td>
<td>1,2-bis-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine</td>
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<tr>
<td>bisPBPC</td>
<td>1,2-bis-(1-pyrenebutanoyl)-sn-glycero-3-phosphocholine</td>
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<tr>
<td>CL</td>
<td>cardiolipin</td>
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<tr>
<td>DE</td>
<td>dielaidoyl</td>
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<tr>
<td>DGalDG</td>
<td>1,2-diacyl-3-O-[α-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl]-sn-glycerol</td>
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<tr>
<td>DG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DGD</td>
<td>Digalactosyldiacylglycerol synthase</td>
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<tr>
<td>DGlcDG</td>
<td>1,2-diacyl-3-O-[α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl]-sn-glycerol</td>
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<td>DGS</td>
<td>digalactosyldiacylglycerol synthase</td>
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<tr>
<td>DO</td>
<td>dioleoyl</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>Glc</td>
<td>glucose</td>
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<td>GT</td>
<td>glycosyltransferase</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MGalDG</td>
<td>1,2-diacyl-3-O-(β-D-galactopyranosyl)-sn-glycerol</td>
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<tr>
<td>MGlcDG</td>
<td>1,2-diacyl-3-O-(α-D-glucopyranosyl)-sn-glycerol</td>
</tr>
<tr>
<td>MGD</td>
<td>monogalactosyldiacylglycerol synthase</td>
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<tr>
<td>MGS</td>
<td>monoglucosyldiacylglycerol synthase</td>
</tr>
<tr>
<td>MAMGlcDG</td>
<td>1,2-diacyl-3-O-[6-O-acyl(α-D-glucopyranosyl)]-sn-glycerol</td>
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<tr>
<td>MADGlcDG</td>
<td>1,2-diacyl-3-O-[α-D-glucopyranosyl-(1→2)-O-(6-O-acyl-α-D-glucopyranosyl)]-sn-glycerol</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
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<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
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<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
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<td>PS</td>
<td>phosphatidylserine</td>
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<td>TM</td>
<td>transmembrane</td>
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One of the most important ingredients for life is a fatty membrane that separates the inside of the cell from the surrounding environment. This means that the cell can have an interior that is chemically different from the outside. Fatty membranes also organize different tasks inside of eukaryotic cells. The membrane consists of a bilayer of lipids (fat) and with membrane proteins embedded into, or attached to the lipids. Membrane lipids are amphiphilic molecules with a hydrophobic tail and a hydrophilic head group. The amphiphilic property of lipids drives the assembly of lipids into bilayer structures. Lipids are generally defined as biological molecules readily soluble in organic solvents such as chloroform, ether or toluene. There is a big variety of membrane lipids with diverse headgroups, acyl chain lengths, grade of saturation and shape. The most common ones are glycerol-based phospholipids or glycolipids. The lipid content also varies greatly in different membranes, for example glycolipids are common in Gram-positive bacteria and in the thylakoid membrane of plants but normally they do not exist in Gram-negative bacteria. Even if there is a big variety of membrane lipids little is known about their specific roles and how they influence different membrane proteins. This is of great interest since 20-30 percent of all proteins are integral membrane proteins and the number gets even higher if also peripheral proteins are taken under consideration. Currently, most medicines are directed towards membrane proteins but in the future, also knowing more about the lipid-protein interactions and lipid synthesizing pathways, the medical targets could be lipids. The focus of this thesis is synthesis of membrane glycolipids as well as how their different properties affect the embedded proteins. A prominent scientist said recently that the last century was the century of the proteins but this will be the century of the lipids!
Lipid-protein interactions

Until recently membrane lipids were simply considered to create the main cell barrier and to be a bulk for membrane proteins to attach to. With more crystal structures of membrane proteins available it is obvious that lipids are more integrated with proteins and might also be considered to act as important cofactors for integral proteins (Ahn et al., 2003; Katona et al., 2003; Roderick et al., 2002; Sazanov et al., 2003). It is steadily becoming clearer that lipids have three different locations in relation to an integral protein; i) either situated in the bulk phase, without any contact with the protein, ii) in an annular shell, tightly surrounding the protein, or iii) at specific sites, often in-between transmembrane α-helices (Lee, 2003).

Annular lipids

It has been demonstrated by a number of approaches that there are annular shells of lipids around transmembrane proteins (reviewed by Lee, 2005). Electron spin resonance (ESR) has shown that a phosphatidylcholine moves in the bulk phase at about $8 \times 10^7$ s$^{-1}$ (Knowles et al., 1979) whereas a lipid attached to a protein exchanges with the bulk at about $1-2 \times 10^7$ s$^{-1}$ (Marsh and Horvath, 1998) both at 30 °C. This means that the lipid-protein interaction is not very strong but still it is stronger than the lipid-lipid interaction. Using ESR one can also determine the number of lipids binding to a membrane protein and it has been shown that this fits reasonably well with the expected circumference of the transmembrane part of the protein in question (Esmann and Marsh, 2006; Jacobs et al., 1997; Jost and Griffith, 1978; Lee, 2005; Taylor and Watts, 1998) Thirdly, ESR spectra of spin-labelled lipids in native membranes show that there is a subpopulation of lipids with more disordered acyl chains than in the bulk phase which would be the case for protein-binding lipids, since they have to fit in with the proteins irregular surface (Jost and Griffith, 1978). Taken together with the fact that many crystal structures show tight binding of lipid molecules, these observations gives us an insight into the close association of proteins and lipids. For example the crystal structure of bacteriorhodopsin shows that there are 18 tightly bound acyl chains surrounding the protein (Luecke et al., 1999).
So what is the driving force of the lipid-protein interaction? One major driving force is hydrophobic matching between the hydrophobic parts of the lipid and the protein (Mouritsen and Bloom, 1993). This is demonstrated by gramicidin A which only forms dimers when there is a good hydrophobic match with the bilayer. In the case of mismatch, where the bilayer is too thick to accommodate a dimer, dimerization can be facilitated by molecules forming curved structures such as cholesterol and lysolipids (Hwang et al., 2003; Lundbaek and Andersen, 1994). The activities of several proteins are also affected by the length of the acyl chains. The active transport of α-galactosides by the *E. coli* melibiose permease is working at its best when the surrounding lipids have an acyl chain length of 16 carbons (Dumas et al., 2000). Other examples of this phenomenon exist for the Ca$^{2+}$-ATPase important for muscle cell action and the Na⁺, K⁺-ATPase that regulates the fine balance of sodium and potassium ions across membranes. The activity of both ion pumps is optimal at a certain chain length; 18:1c and 22:1c respectively. Interestingly, when cholesterol is added to the membrane the optimal chain length for Na⁺, K⁺-ATPase gets much shorter, indicating the tendency of cholesterol to thicken the membrane, which then will be compensated by the shorter lipids (Cornelius, 2001; Lee, 1998). Moreover, lipid protein simulations have shown that the main interactions energies between lipid and protein appear to consist of nearly constant weak van der Waals and
electrostatic interactions (Woolf and Roux, 1996). If stronger interactions as hydrogen bonds are present one should instead consider the more specifically binding lipids.

**Co-factor lipids**

There are lipids that are preferred by a protein, meaning that the binding constants are higher for a certain kind of lipid than for other at specific areas of the protein. The mechanosenstive channel MscL of *Mycobacterium tuberculosis* has a single class of binding sites which have a preference for anionic lipids such as cardiolipin, phosphatidylglycerol and phosphatidylserine. These anionic lipids have about twice as high binding constants compared to neutral phosphatidylcholine. The binding decreases with higher ionic strength suggesting that charge interactions are important for binding. Also MscL has higher binding of phosphatidic acid at two classes of binding sites, but these are less dependent on ionic strength showing that other interactions are important for binding (Powl et al., 2003). In the photosynthetic reaction center from *Thermochromatium tepidum* the zwitterionic phosphatidylethanolamine binds in a specific manner. Binding to the protein changes the conformation of PE compared to bulk phase, allowing the phosphate group to interact with adjacent Lys and Arg groups (Fathir et al., 2001). The selectivity at a site of this sort will depend on the headgroup size, on the charge and on the grade of hydrogen bonding possible for the lipid (Lee, 2005). In the reaction center of *Rhodobacter spheroides* a molecule of the anionic lipid cardiolipin is tightly integrated. X-ray diffraction data collected over the resolution range 30.0-2.1 Å show that binding of the lipid to the protein involves a combination of ionic interactions between the protein and the lipid headgroup and van der Waals interactions between the lipid tails and the membrane-spanning surface of the protein. In the headgroup region, ionic interactions involve polar groups of a number of residues, the protein backbone, and bound water molecules (McAuley et al., 1999). Mutagenesis of a highly conserved arginine (to leucine) that binds to the head-group of the cardiolipin did affect the thermal stability of the protein, showing an approximate 5 °C degrees decrease in melting temperature. It was proposed that one function of the cardiolipin is to stabilise the interaction between adjacent membrane-spanning alpha-helices in a region where there are no direct protein-protein interactions (Fyfe et al., 2004).

Another way to study the specificity of certain lipids to different proteins or functions is to knock out genes encoding for lipid synthesizing proteins, and thereby reducing or removing a particular lipid in an organism (Dowhan et al., 2004). This will be further discussed in later chapters.
Peripheral proteins- association to membranes

Lipid-protein interactions are not exclusive to membrane spanning proteins, as the peripheral proteins also interact with lipids. Lipid membranes generally carry a significant negative charge due to anionic lipids such as phosphatidylserine, phosphatidylglycerol and cardiolipin. The most important long distance force targeting peripheral proteins is therefore electrostatic attractions between cationic residues of the protein and the anionic membrane. Closer association is due to the energy reduction followed by loss of polar aqueous solvent and to the ability to bury hydrophobic residues into the bilayer. This will also direct the protein into the correct orientation to the membrane (Mulgrew-Nesbitt et al., 2006). Numerous peripheral proteins do not show any activity at all without anionic lipids present because they remain in the cytosol (Li et al., 2003; Lomasney et al., 1999; Wikstrom et al., 2004).

There are also more specific interactions with particular headgroups and often combinations of all types of interactions. Specificity for phosphatidylserine has been shown for the eukaryotic phospholipase PLC-δ1 catalyzing the hydrolysis of PIP$_2$ yielding diacylglycerol (DAG) and the second messenger IP$_3$. PLC-δ1 was activated 20-fold by anionic phosphatidylserine (PS) compared to only 4-fold by anionic phosphatidic acid (PA) and not activated at all by zwitterionic phosphatidylethanolamine (PE) and neutral phosphatidylcholine (PC) (Lomasney et al., 1999). This difference in activation indicates the specificity for PS since PA, even though anionic, only gives a very low activity of the enzyme.
Nonbilayer-prone lipids

Nonbilayer-prone lipids exist in all biological membranes. These lipids would by themselves not form a bilayer but would most often build, so-called hexagonal or cubic structures (of the reversed type), with the acyl chains pointing outwards and the headgroups inwards. These rod-like structures are organized into a hexagonal packing and thereby the name. The nonbilayer-prone lipids are also referred to as reversed hexagonal- or non-lamellar-forming lipids. Bilayer-forming lipids such as PC or diglycosyldiacylglycerol (DGDG) exist in two phases either in ordered gel (Lβ) or in liquid crystalline (Lα) phase, whereas nonbilayer-prone lipids exist at low temperatures in Lβ, at intermediate temperatures in Lα and in high temperatures in cubic or hexagonal (HII) phase (Seddon et al., 1983). Moreover, a membrane containing nonbilayer-prone lipids has usually a higher transition temperature for the Lβ to the Lα phase (Caffrey and Hing, 1987).

**Figure 2.** Phases and molecular shapes of diverse lipids (Dowhan and Bogdanov, 2002).
Nonbilayer prone lipids have relatively small headgroups and can change from cylindrical to conical shape with increasing length of acyl chains and unsaturation. Furthermore, environmental factors as temperature, pH, salt concentration and the presence of divalent cations and hydration, can change the overall lipid shape (Tate et al., 1991; van den Brink-van der Laan et al., 2004). The presence of divalent cations in a monolayer decreases the molecular surface area of cardiolipin (in the order Ca\(^{2+}\)/Mg\(^{2+}\)>Sr\(^{2+}\)>Ba\(^{2+}\)), which thereby makes CL more nonbilayer prone (Killian et al., 1994). Also both phosphatidylserine and phosphatidic acid form hexagonal phases at pH:s below 4 and 3, respectively (de Kroon et al., 1990; Verkleij et al., 1982). The cell membranes of *Acholeplasma laidlawii* and *E.coli* are regulated to maintain a physical state of the membrane lipids close to a bilayer-nonbilayer phase transition state by changing either the headgroups (*A. laidlawii*) or the acyl chains (*E. coli*) (Lindblom et al., 2002; Morein et al., 1996; Vikstrom et al., 2000). The two main nonbilayer prone lipids in especially bacterial membranes are phosphatidylethanolamine (PE) and monoglycosyldiacylglycerol (MGDG), while other lipids are not considered nonbilayer prone under normal conditions.

### Membrane effects

Nonbilayer-prone lipids have several effects on the physical state of membranes. If you imagine lipids with a smaller headgroup area than occupied by the acyl chains, they are conically shaped. If you now put them together in a row they will not form a straight line but would bend upward i.e. negative spontaneous curvature (from a membrane perspective). The tendency to bend is quantitatively described by the intrinsic radius of curvature, \(R_0\), which minimizes the bending energy of a lipid monolayer. When bilayer (large \(R_0\)) and nonbilayer lipids (small \(R_0\)) are suitably mixed, the resulting layer has a value of \(R_0\) that is critical for bilayer stability (Gruner, 1985).

When the nonbilayer-prone lipids are “forced” into a bilayer they will be under stress. The stress that this will cause on the membrane is often refereed to as curvature stress or packing stress (Nakano et al., 2005). Curvature stress can be proven indirectly for example by changes in the \(L_{α}\) to \(H_{II}\) transition temperature (Janes, 1996). This stress can be thought of as a form of force that might provide energy for conformational changes of transmembrane protein segments. One clear example of this force is that rhodopsin changes conformation to an active state when surrounded by nonbilayer-prone lipids (Botelho et al., 2002). The curvature stress can also be looked upon in another way, that the stress is causing a change in the lateral pressure profile within the membrane. The lateral pressure varies with depth in the membrane (Cantor, 1997). Adding nonbilayer-prone lipids to a lipid mixture contributes to a decreased headgroup pressure and an increased chain
pressure (Bezrukov, 2000). In both monolayers and vesicles the catalytical domain of leader peptidase inserts more efficiently when smaller headgroups are present and the binding decreased when increasing the headgroup sizes (van den Brink-van der Laan et al., 2001). This indicates that the lateral pressure is lower in the interphase with nonbilayer prone lipids present in the membrane.

Other membrane changes related to nonbilayer prone lipids are hydrophobic mismatch and packing defects. The hydrophobic mismatch between lipids and proteins previously discussed might be easier to accomplish in a local environment with negative curvature. The packing defects are suggested to be introduced when a bilayer undergoes gel to liquid (L β to L α) transition and would give “hydrophobic spots” on the surface of the membrane. These spots could influence the binding of peripheral proteins (van den Brink-van der Laan et al., 2004c). All the discussed effect that nonbilayer prone lipids have on the membrane could clearly influence both transmembrane and peripheral proteins in different ways.

**Influence on membrane proteins**

It has been shown that nonbilayer prone lipids affect the assembly, folding, stability and activity of transmembrane proteins as well as the activity and binding of peripheral proteins (van den Brink-van der Laan et al., 2004c). It is not always clear what changes caused by nonbilayer lipids influence the membrane protein, but it seems that the curvature stress which also affects the lateral pressure provides a reasonable answer for studied effects. In a natural membrane there is a mix of nonbilayer-, bilayer-, anionic- and neutral lipids, which must be taken under consideration when studying protein activity.

**Transmembrane proteins**

There are numerous membrane spanning proteins affected in different ways by the physical state of the membrane. For the bacterial translocase SecYEG, the nonbilayer lipid PE strongly stimulates the activity. However, only to a certain extent, for both *E. coli* and *B. subtilis* secYEG the optimal activity is obtained when the lipid content matches the natural membrane with a mixture of nonbilayer, anionic and neutral or zwitterionic lipids (van der Does et al., 2000). The mitochondrial adenine nucleotide transporter has its highest activity at a radius of curvature R o which corresponds to a ratio of DOPC:DOPE 5:3 which is almost exactly the R o for the inner mitochondrial membrane (Streicher-Scott et al., 1994).

One way to determine whether the studied affect really is due to the nonbilayer prone tendency is to change the acyl chains so that the lipid becomes
more bilayer prone. This was done in studies on the ubiquinol-c reductase, where DOPE was changed to DEPE. This configuration of the double bond in the acyl chains results in a much higher $L_{\alpha}$ to $H_{II}$ phase transition temperature (from 13 °C to 65 °C). The respiratory control ratio was barely affected by the DEPE or PC, CL, PI and PA (all bilayer prone lipids), whereas with DOPE it was highly stimulated (Yang and Hwang, 1996).

Another approach is to include both MGDG and PE, both nonbilayer prone lipids, to study if they can recover the activity of an enzyme. This has been done for the $E. coli$ lactose permease (LacY) both for topology studies and energy-dependent transport. PE, MGlcDG and PC (all neutral in membranes) but not anionic PG nor anionic CL can support correct topology of the transporter whereas only PE and MGDG can support active transport (Xie et al., 2006; Paper III). This shows the importance of neutral lipids for the topology and of nonbilayer prone lipids for the activity of LacY. A similar approach was taken with the ATP-driven $Ca^{2+}$ of the sarcoplasmic reticulum where it was demonstrated that the ATP-driven $Ca^{2+}$ uptake and coupling ratios were progressively increased when the molar ratios of DOPE/DOPC and MGalDG/DOPC in vesicles were increased (Navarro et al., 1984). Also for rat liver GDP mannose-dolicholphosphate mannosyl transferase the optimal activity was found in the presence of PE or MGalDG (Jensen and Schutzbach, 1988).

By introducing compounds that promote the hexagonal phase, such as different alcohols or cholesterols (Wieslander et al., 1986), makes it possible to investigate the physical state dependence membrane proteins. Extensive studies on the Streptomyces lividans KcsA potassium channel have been done concerning lipid effects. The tetramer stability of the channel increases with PE but not with PC or PG (van den Brink-van der Laan et al., 2004b 518). Results of adding different alcohols with different affects on the phase transitions also indicate that the lateral pressure is the important factor for channel assembly; alcohols promoting the bilayer phase even dissociated the tetramer (van den Brink-van der Laan et al., 2004a).

These studies demonstrate the importance of a correct physical state of the membrane with a large portion of nonbilayer prone lipids for transmembrane proteins. Moreover, these studies also show that this dependence is consistent in many organisms, types of cells and membranes.

Peripheral proteins

The way that a peripheral protein is influenced by the physical state of the membrane should obviously differ from the TM proteins. However, the altered lateral pressure induced by nonbilayer prone lipids also affects the headgroup region and thereby the main region to which peripheral proteins attach. There are numerous examples of peripheral proteins also dependent on nonbilayer prone lipids.
The activity of the peripheral CTP:phosphocholine cytidylyltransferase, a rate-limiting enzyme in phosphatidylcholine biosynthesis, is modulated by its interaction with lipid bilayers (Kent, 1997). The regulation of this enzyme is of central importance in the maintenance of membrane lipid homeostasis. Its activity is clearly more enhanced with PE lipids compared to PC lipids in monolayer studies (Attard et al., 2000). Also protein kinase C is stimulated by changes in curvature stress, and interestingly different isoforms have different optimal values of curvature stress (Stubbs and Slater, 1996). Protein kinase C is activated by nonbilayer-prone PE (Bazzi et al., 1992), and also by drugs which promote the conversion of phospholipid bilayers to the hexagonal phase. In contrast, drugs that stabilize the bilayer phase are inhibitors of protein kinase C (Epand, 1987). A combination of using both DOPE/DEPE and eicosane (which promotes hexagonal phases) was used with mitochondrial cytochrome P450SCC, giving evidence that the phase behavior was crucial for proper activity (Schwarz et al., 1997). Digluco-syldiacylglycerol synthase (DGS) from *A. laidalwii* has a contradictory behavior considering activation by hexagonal H_{II} phases. It is stimulated by DAG, DOPE and cholestenone but inhibited by MAMGlcDG and MADGlcDG though they all contribute to nonbilayer tendency. This indi-
cates a fine-tuning task of this protein considering the physical state of the membrane, by the production of the bilayer prone lipid DGlcDG (Li et al., 2003; Vikstrom et al., 1999).

Many peripheral proteins bind stronger to the membrane in the presence of non-bilayer prone lipids. The G-proteins Gαi and Gαs, protein kinase C and apolipophorin III bind better to liposomes with PE than with PC (Escriba et al., 1997), and aMGS has higher binding constants with PE and DAG, shown in Biacore experiments (Li et al., 2003). It should be stated however that the binding to nonbilayer prone lipids and enhanced activity do not always go hand in hand for peripheral proteins (Dahlqvist et al., 1995; Li et al., 2003). This signifies that binding is not sufficient for optimal activity of peripheral proteins but that their orientation also are important.

AD93- an E. coli strain lacking phosphatidylethanolamine

Phosphatidylethanolamine (PE) is the major lipid in E. coli and accounts for 70-80 percent of the total amount of glycerophospholipids in the membrane. It exists in both layers of the inner membrane and is slightly accumulated in the inner layer of the outer membrane. PE is zwitterionic, but neutral in the membrane, and also a nonbilayer prone lipid (Mantsch et al., 1981). The other main lipids are phosphatidylglycerol (PG) and cardiolipin (CL) which are both anionic lipids. Moreover, CL is induced to form hexagonal HII phase with divalent cations (Killian et al., 1994).

William Dowhan and associates have created a PE deficient E. coli strain that almost completely (0.007 %) lacks PE. This strain was constructed by inactivating the gene encoding the phosphatidylserine synthase (pss) which catalyzes the step prior to the synthesis of PE (DeChavigny et al., 1991). Initially, cell growth was maintained by introducing a plasmid carrying the pss gene which was thereafter removed by curing. Cell growth stopped when phosphatidylethanolamine reached below 30 mole %, but divalent metal ions such as Ca2+, Mg2+ and Sr2+ could rescue growth. This is believed to depend on the fact that CL turns more nonbilayer prone with divalent cations present as mentioned earlier (Killian et al., 1994), and therefore CL might be able to replace PE and correct for the loss of a proper physical state of the membrane. This hypothesis was supported by the fact that AD93 cells adapt to the lipid composition depending on the type of divalent cations present. Ca2+ was found to be a stronger inducer of the inverted hexagonal phase than Mg2+ and this is compensated by a lower level of cardiolipin (Rietveld et al., 1993). The membrane of this PE lacking strain AD93 (when grown in 10 mM MgCl2) contains app. 85 % PG and 15 % CL, both anionic lipids. The
acyl chain composition on the other hand is almost unchanged in this strain compared to wild type (DeChavigny et al., 1991).

This strain has plenty of altered characteristics and surely some unknown ones. The most obvious phenotype are very filamentous cells, partially due to mislocalization or misassembly of the FtsZ-dependent division machinery (Mileykovskaya et al., 1998). Also MinD, taking part in directing the Z-ring to the correct division site, misassembled into clusters (Mileykovskaya et al., 2003). Another apparent phenotype of the pss mutant is its lack of motility, due to that the flagellar genes are not transcribed in the pss mutant (Shi et al., 1993). Also interesting, the Cpx stress response pathway is always activated in these cells, indicating a reaction to altered physical property of the membrane (Mileykovskaya and Dowhan, 1997). The pss lacking cells are also highly sensitive to both high temperatures (DeChavigny et al., 1991) and acid shock (Canet et al., 2003). The long time survival at stationary phase is highly affected, after just 1 day the cell viability for AD93 is down to 20 % compared to 100 % for wild type cells. After 5 days all PE minus cells are dead. Wild type cells however are app. 90 % viable after 7 days and longer (Canet et al., 2003). Three enzymes in the sugar phosphotransferase system, the glucose enzyme II, the mannose enzyme II, and the mannitol enzyme II are all almost completely inhibited by the lack of PE in vivo (Aboulwafa et al., 2004).

Studies on three different membrane transporters have shown altered topology in PE lacking cells. For lactose permease the first six transmembrane segments are completely reversed (Bogdanov et al., 2002; Zhang et al., 2005). Since the topology is shifted for these three transporters in AD93 the active transport carried out by these proteins are also reduced (Zhang et al., 2003) (Bogdanov and Dowhan, 1995; Zhang et al., 2005), most drastically for the Lactose Permease which has only about 10 % of normal lactose uptake in PE lacking cells (Bogdanov and Dowhan, 1995).

However for all these altered characteristics and effects on membrane proteins one has to consider at least three possible explanations; either the changes are due to i) a specific demand of zwitterionic PE; ii) the loss of the nonbilayer tendency of PE; or iii) that the surface charge is completely altered in these cells. It has been shown in vivo that both for topology and activity of the Lactose Permease the MGlcDG lipid can replace PE. As previously mentioned PC can also correct the topology but not the activity (Xie et al., 2006). This indicates that the physical state which both MGlcDG and PE lipids introduce to the membrane is important for the activity of this transporter.
Glycolipids

Glycolipids have been found in bacteria, archebacteria, yeast, algae, higher plants and in animals. They are mainly building blocks in many diverse membranes but are also known to be important for example in cellular recognition and immune response events. The term glycolipid stands for a compound containing one or more monosaccharide residues bound by a glycosidic linkage to a hydrophobic moiety such as an acylglycerol, a sphingoid, a ceramide (N-acylsphingoid) or a prenyl phosphate. They can be divided into three classes; glycolipids with a glycerol backbone, glycosphingolipids (GSP:s) which are ceramide-based sphingolipids, and glycosphingolipids (GPI:s) with DAG backbone. GSLs are distributed among all eukaryotic species and are also found in some bacteria. In eukaryotic cell surfaces they can act as both the primary interface between bacteria and their host and secondly as a targeting mechanism for bacterial virulence factors. GPIs only exist at the surface of eukaryotic cells where they can attach to the C-terminus of a protein during posttranslational modification. Thereafter they have a diverse function ranging from enzymatic to antigenic and adhesion. In animals the main glycolipids are glycosphingolipids but animals also possess glycolipids in specific tissues. The glycoglycerolipids are building blocks of membranes in both prokaryotes and eukaryotes and are the central focus of this section.

The main membrane glycolipids are diacylglycerol (DAG)-based and they exist in Gram-positive bacteria, in plastids of plants, and some specialized animal cells. They are rare in Gram-negative bacteria but an exception to this is that they exist in photosynthetic Gram-negative bacteria. They have different sugar moieties such as glucose, galactose and mannose which are isomers of the same sugar. In bacteria the glyco-headgroups can be all three isomers and there is a big variety of mono-di-tri and even- polyglycosyldiacylglycerols where the sugars bind in different configurations of either α or β bonds. The thylakoid membranes of plants on the other hand consist basically of four major components: monogalactosylDAG, digalactosyl-DAG, sulfoquinovosylDAG and phospholipids; the major ones being the galactolipids. In animal tissues a minimum of six galactolipids are found mostly located in nervous tissue and/or testis and spermatozoa. These six can be divided into two subclasses galactosyldiacylglycerols and galactosylyacylalkylglycerols which both contain a monogalactosyl moiety, a sulfated monogalactosyl moiety and a digalactosyl moiety. (Kates, 1990).
Physical properties of Monoglycosyldiacylglycerol and Diglycosyldiacylglycerol

As mentioned in earlier sections the nonbilayer tendency of a lipid is important for proper function of numerous transmembrane and peripheral proteins. A phase diagram of dioleoyl-MGlcDG-H\textsubscript{2}O was set up using NMR spectroscopy and polarized light microscopy. This diagram shows that already at temperatures above 10°C only non-lamellar phases exist (Lindblom et al., 1986). This is comparable to the lipid PE, which as MGDG is a biologically common lipid, where the dioleoyl-PE undergoes lamellar-nonlamellar phase transition at approximately 10 °C (Tilcock and Cullis, 1981). On the other hand α-MGlcDG and its β-linked counterpart with straight, saturated acyl chains are much more prone to form nonlamellar phases than the corresponding PE (Mannock et al., 1990). Moreover, the nonbilayer tendency increases for MGlcDG with both increasing length and degree of unsaturation of the acyl chains (Rilfors et al., 1993). MGalDG from plants that has two highly unsaturated fatty acyl chains is thereby considered to be a potent non-bilayer forming lipid (reviewed by Lee, 2000). Adding a sugar, producing the DGlcDG lipid variant gives a larger headgroup and thus DGlcDG lipids have not been shown to form any nonlamellar phase in any variants or under any conditions (Rilfors et al., 1993). One also have to consider that the nonbilayer tendency can vary not only with temperature, chain length and degree of saturation, but also with pH, cations, hydration and other molecules including other present lipids (Rilfors et al., 1984). All these factors can change the look of a phase diagram towards more nonbilayer prone- or bilayer prone- features.

![Figure 4. Molecular structures of:](image)

A. α-monoglucosylDAG  
B. β-monogalactosylDAG  
C. α-diglucosylDAG  
D. αβ-digalactosylDAG  

from A. laidlawii and plants
The degree of hydration is also considered to be an important physical factor of lipids, mainly to sustain a functional permeability barrier. The maximum hydration at 35 °C of MGlcDG in the reversed hexagonal phase increases from 7 to 11 moles water/ moles lipid when varying the acyl chain length from 14 to 18 (cis-unsaturated) Here the DGlcDG variant is almost similar also varying in between 7 to 11 moles water/ moles lipid under the same conditions (Lindblom et al., 1986). This shows that an increase in acyl chain length increases the water binding capacity. The hydration capacity also increases with increasing temperature and in the presence of divalent cations (Wieslander et al., 1978). There is no exact comparison in hydration capacity to DOPE but at 26 °C the water to lipid molar ratio varies from 8 to 14 with different acyl chains (Rilfors et al., 1994). Comparing this to DOPC which under the same conditions takes 30-34 moles water/ moles lipid (Rilfors et al., 1993) gives an idea that the MGlcDG and DGlcDG are closer to DOPE in hydration capacity than to DOPC. To compare the glucose-DAG lipids with the galactose-DAGs we have to consider the percentage of hydration. For the glucose-lipids the hydration is approximately 13 % (w/w) whilst the galacto-lipids have a hydration capacity of app. 22 % (w/w) (Wieslander et al., 1978) which is considerably higher.

The area occupied by MGlcDG and DGlcDG in the air/water interphase has been compared using x-ray diffraction studies. Varying the acyl chain length gives small differences but the MGlcDG size is app. 60 Å² and the DGlcDG size is app. 70 Å² (Eriksson et al., 1991). Even if the headgroup for DGlcDG is larger than that of MGlcDG, which is more non-bilayer prone, the headgroup size can not be directly translated to nonbilayer tendency. The headgroup of PE is smaller than for MGlcDG but nevertheless MGlcDG are more prone than PE, if both have saturated acyl chains, to form non-bilayer structures (Rilfors et al., 1993). There is no study using all four mono and di /gluco- and galacto- DAG lipids for comparing the headgroup sizes with identical acyl chains. But interestingly, molecular models of both diglucosyl and digalactosyl-DAG lipids shows a remarkable difference in the appearance of the headgroups. The second sugar on the DGalDG is linked with α 1→ 6´ and becomes rather flexible compared to the ones with α 1→ 4´ and α 1→ 2´ linkages. This flexibility makes the headgroup of DGalDG occupy a larger area than the two Glc in the DGlcDG variant which lies more flat against each other and the membrane (Iwamoto et al., 1982).

Glycosyltransferases

Glycosyltransferases (GTs) catalyze the transfer of monosaccharide moieties to a variety of biologically substrates as proteins, lipids, carbohydrates and nucleic acids. Their products are often vital for survival of the organism. GTs also belong to one of the largest and diverse enzyme group and exist in
all living cells. Considering the variety of acceptors and donors that the glycosyltransferases can use, it is easy to believe that there would be a large variety of structural folds for this family. Nevertheless, so far only two different folds have been crystallized, named the GT-A fold-group and the GT-B fold-group (Breton and Imberty, 1999) with new structures belonging to both groups turning up as this is being written (Grizot et al., 2006; Jinek et al., 2006). Bioinformatic tools also predict that there are two other folds, the GT-C family and, unique for eukaryotes, the GT-D family (Kikuchi et al., 2003).

All glycosyltransferases contain a distinct donor and acceptor binding site connected by a linker region which forms the active site (Klutts et al., 2006). The GT-A family has two dissimilar and smaller domains. This fold consists of an N-terminus which is composed of several beta-strands that are flanked by alpha-helixes forming a Rossman like fold and a C-terminus which is composed mainly of Beta-sheets (Qasba et al., 2005). These domains are tightly integrated with each other and thereby form a quite globular protein compared to the GT-B fold which consists of two distinct domains. These two domains are both Rossman folds where the acceptor molecule binds to the N-domain whereas the C-domain binds to the sugar donor (Qasba et al., 2005).

The transfer of the glycosyl moiety from one molecule to another occurs either in an inverting or retaining mechanism and both the GT-A and the GT-B fold use both mechanisms. In inverting glycosyltransferases, the deprotonated hydroxyl group of the acceptor attacks the C1 anomeric carbon of the sugar donor to form a glycosidic bond, resulting in an inversion of the configuration at C1. For the retaining mechanism the glycosidic bond is formed between the donor and acceptor, but how the retaining to the C1 configuration works is less clear. The glycosyltransferases often demands metal ions as cofactors and binding of these results in a conformational change of the protein. This conformational change, in which a loop acts as a lid covering the bound donor substrate, creates an acceptor binding site. For both mechanisms when the glycosyl moiety has been transferred the saccharide product is ejected and any remaining product is released (Qasba et al., 2005).

**Acholeplasma laidlawii**

*A. laidlawii* belongs to the group of mycoplasmas which are the smallest free-living organisms known, with a diameter of only 0.5-1 µm. The genome of *A. laidlawii* is about 1600 kb and about 200 membrane proteins can be visualized on a 2D-gel (Weisburg et al., 1989; Wieslander et al., 1982). It has only one membrane and also lacks a cell-wall, which makes it possible to easily vary the membrane properties and therefore it is an excellent model for various membrane studies. The major lipids in *A. laidlawii* are under most
conditions the two glucolipids MGlcDG and DGlcDG. MGlcDG is a neutral, nonbilayer-prone lipid which is crucial for bilayer packing properties in the *A. laidlawii* membrane and varying in amounts from 5 to 50 mole% depending on growth conditions. DGlcDG is a neutral, but bilayer forming glucolipid. These two lipids are jointly regulated to give a constant radius of spontaneous curvature, including similar bilayer/nonbilayer transition temperatures and also a defined membrane surface charge (Lindblom et al., 1986; Österberg et al., 1995).

**Figure 5.** Major lipid biosynthesis in *A. laidlawii*. The cone and the cylinder represent the packing shapes of MGlcDG and DGlcDG respectively.

There are two enzymes synthesizing these major glucolipids; the monoglucosyl diacylglycerol synthase (alMGS) and the diglucosyl diacylglycerol synthase (alDGS). These two glucosyltransferases use UDP-glucose as a sugar donor and catalyze the glucosylation of DAG and MGlcDG respectively (Dahlqvist et al., 1992). They are both membrane associated proteins, probably both by electrostatic and hydrophobic interactions (Berg et al., 2001; Edman et al., 2003; Karlsson et al., 1997). Both alMGS and alDGS require a fraction of anionic lipids for activity, as for example phosphatidylglycerol (PG) (Karlsson et al., 1994) (Karlsson et al., 1997). The alDGS demands PG more specifically than any other anionic lipid (Vikstrom et al., 1999). The demand of anionic lipids is probably due to that both enzymes
have a highly positively-charged N-terminus domain which should preferentially bind anionic lipids (Berg et al., 2001; Edman et al., 2003). Not only the strength of binding affects the activity; binding of MGS in a proper orientation/conformation seems most important for activity indicating that the active site should be correctly placed in relationship to the membrane (Storm et al., 2003). They are also both stimulated by a small amount of nonbilayer-prone lipids but αDGα to a higher extent and is therefore looked upon as the strongest regulating enzyme considering membrane curvature (Li et al., 2003; Vikström et al., 1999). The functional similarities between these enzymes are due to that both enzymes appear, on the basis of sequence and structural modeling, to belong to the same structural superfamily (GT-4). This structural family, which belongs to the GT-B fold, includes the known structures of membrane bound glycosyltransferase MurG and the soluble GlcNAc epimerase (paper II).

**Streptococcus pneumoniae**

*S. pneumoniae* belongs to the group streptococci, that are Gram-positive cocci found in the normal flora of humans, as well as species that can cause both mild and life threatening diseases. *S. pneumoniae* is responsible for diseases such as pneumonia, sepsis, ear infection and meningitis and is one of the leading causes of mortality in the world. A large number of multi-antibiotic-resistance strains are widely spread (Gilmore and Hoch, 1999; Novak et al., 1999). The main virulence factor in *S. pneumoniae* is the polysaccharide capsule, the function of which mostly prevents polymorphonuclear leukocytes from engulfing and digesting the bacteria (Garcia et al., 1997). *S. pneumoniae* comprises 90 serotypes, each one having a unique polysaccharide capsule. The first complete genome of app. 2.2 M base pairs from a virulent isolate was sequenced in 2001 (Tettelin et al., 2001).

The membrane of *S. pneumoniae* consists mainly of the two glycolipids MGlcDG and DGlcGalDAG and a small fraction of phospholipids, PG, CL and PC (Szabo et al., 1978). Little work has been done on the glycosyltransferases taking part in the synthesis of these glycolipids. Nonetheless, these glucosyltransferases are also believed to belong to the same structural family as the GT:s from *A. laidlawii* (paper I and II). Additional similarities/dissimilarities of the glycosyltransferases from these two organisms will be further discussed in the result section.

**Arabidopsis thaliana**

In plant green tissues, in contrast to animal and yeast, the most common lipids are galactolipids and not phospholipids. They contribute to about 75 percent of the total membrane lipids in leaves and are therefore the most
abundant lipid class in the biosphere. The galactolipids are greatly enriched in plastids and thus also in the thylakoid membranes, where all the photosynthetic complexes including ATPase are located (Dörmann and Benning, 2002). In pea, as an example, the thylakoid membrane consists of nearly 90 percent of MGalDG and DGalDG, whilst the minor lipids being sulfoquino-

vosyldiacylglycerol, phosphatidylglycerol and phosphatidylcholine (Schmid and Ohlrogge, 2002). The crystallization of the PSI complex from the ther-

mophilic cyanobacterium *Synechococcus elongatus* revealed that each monomer contains four lipid molecules, three molecules of PG and one molecule of MGalDG (Jordan et al., 2001). Furthermore the crystal structure of the light-harvesting complex of the dinoflagellate *Amphidinium carterae* contained two molecules of MGalDG (Hofmann et al., 1996). Isolation of the LHCII from peas chloroplast shows the presence of both PG and DGalDG lipids. DGalDG was proven to be essential for the crystallization of 3D crystals and large 2D crystals whereas it had no effect on the oligomeri-

zation of the complex (Nussberger et al., 1993). These results clearly show the close interactions between these photosynthetic complexes and their surrounding galactolipids. For further evidence of this we will look at some results from lipid mutants of *Arabidopsis thailiana*.

**Figure 6.** Galactolipids in the thylakoids and in some of the photosynthetic complexes (Dörmann and Benning, 2002).

*A. thailiana* is a flowering plant related to cabbage and mustard. It is the major model for studying plants due to a relatively small genome size of about 25 million base pairs and a rapid life cycle of about 6 weeks from germination to mature seed. It was also the first sequenced plant genome in 2000 (Tabata et al., 2000). There are at least three genes encoding for the MGalDG synthases (MGD1, MGD2 and MGD3) in *Arabidopsis* (Awai et al., 2001; Jorasch et al., 2000; Miege et al., 1999). The main MGalDG syn-

thase is the MGD1 (Jarvis et al., 2000) that as judged from structural model-
ing resembles both the aMGs and aDGs (Botte et al., 2005), paper II). This enzyme catalyzes the synthesis of MGalDG from diacylglycerol and UDP-galactose and is situated in the inner envelope of the plastids (Miege et al., 1999). The MGD2 and MGD3 have a much shorter N-terminal amino acid sequence and no detectable targeting sequence. They are therefore suggested to belong to a different subfamily than MGD1 (but in the same CAZY GT-family nr. 28) and they are also believed to, in contrast to MGD1, localize in the outer envelope of the chloroplasts (Awai et al., 2001). It is clear the MGD1 is the major synthase of MGalDG since a 75 % decrease in transcription level gives the same reduction of the MGD1 lipid. This mutant also gives a reduction of 50 % in chlorophyll level and striking effects on chloroplasst fine structure. This mutant plant is also defective in growth (Jarvis et al., 2000).

Arabidopsis is believed to possess two different DGalDG synthases refereed to as DGD1 and DGD2. The DGD1 produces most of the DGalDG lipids since a mutation of this gene reduces the lipid product level of 90 % (dgd1) (Dörmann et al., 1995). DGD2, which is a parologue of DGD1, has been shown to produce DGalDG by expression in E. coli (Kelly and Dörmann, 2002). Similar to mgd1 mutants the dgd1 mutants give a decrease in chlorophyll levels, and also a clear change in the fine structure of the chloroplast and a severe growth defect (Dörmann et al., 1995). Furthermore, different components of the photosynthetic electron transport chain as well as the structural integrity of the water-oxidizing complex are affected in the dgd1 (Reifarth et al., 1997). There are changes in amounts of photosynthetic complexes PSI, PSII cytochromes b559, D1 and in the xantophyll water-cycle activity (Hartel et al., 1997). Another interesting mutant of Arabidopsis is one where they have inserted the βGlcT gene of Chloroflexus into the dgd1 mutant. This strain produces about 18 percent of βGlcβGalDG lipid (this is almost the same as wild type amounts of the αGalβGalDG) which then almost recovers the growth defect of the dgd1 mutant. However this lipid does not fully support maximal photosynthetic efficiency. Jointly these findings give an insight to the significance of galactolipids for the thylakoid membrane structure and the photosynthetic protein or protein complexes therein.

The MGD1 and the DGD1 enzymes are also suggested to be regulated to achieve a curvature in a certain biologically acceptably range as the analogous enzymes in A. laidlawii (Dörmann and Benning, 2002). The Arabidopsis MDG and DGD enzymes are by no means as well studied for regulation as the A. laidlawii enzymes. However, unpublished data shows that the Arabidopsis enzymes are also stimulated by certain anionic lipids; MGD1 and DGD2 show dissimilar stimulation by different anionic lipids. Also the binding of these enzymes, according to Biacore binding studies, is higher with anionic lipids. Furthermore these binding patterns were also different comparing MGD1 and DGD2 (Kelly, A.A. unpublished data).
Escherichia coli is without any competition the most well studied bacterium in science. It is a Gram-negative bacterium with two lipid membranes, the inner and the outer, and a cell wall composed of peptidoglycan in the periplasmic space. E.coli is commonly found in the human gastrointestinal system where it is harmless but there are also pathogenic strains that can cause severe stomach diseases. The non-pathogenic strain K12 was first sequenced in 1997 and it has a genome size of app. 4.6 Mbp (Blattner et al., 1997). The relatively large genome together with the relative advanced membrane makes E.coli a good model for other larger and more complex organisms according to certain basic rules of life.

Figure 7. The membranes of Escherichia coli in green. This picture is in natural proportions. (© David S. Goodsell 1999)

The inner membrane of E. coli consists of app. 70-80 % of PE, and the ratio of PG and CL varies with strain and environment. PE is somewhat accumulated in the inner leaflet of the outer membrane, but otherwise the inner leaflet has the same lipid composition as the inner membrane (Dowhan, 1997). The outer monolayer of the outer membrane consists mainly of Lipopolysaccharide (LPS). LPS consists of three moieties: the hydrophobic lipid A, which anchors it to the outer membrane; a generally well-conserved...
surface-exposed hydrophilic oligosaccharide, designated the core; and a
hypervariable polysaccharide, the O-antigen (Jann and Jann, 1987). The
membrane associated functions that will be discussed in this section involve
both the inner and outer membrane of *E. coli*. These functions, including
permeability, cell division and osmotic stress tolerance, are all essential for
survival of the *E. coli* cell. Furthermore the topology of transmembrane pro-
teins in the inner membrane (IM) will be discussed. How membrane lipids
are involved in these functions will be one focus of this section.

Permeability

Is it essential for all cells to keep certain chemicals out and others accumu-
lated inside. In Gram-negative bacteria the permeability barriers consist of
the outer membrane with an extensive amount of lipopolysaccharides (LPS),
the cell wall and the cytoplasmic membrane. LPS are a major factor in keep-
ing large hydrophobic molecules out, and it has been shown that a larger
amount of phospholipids in the outer leaflet of the outer membrane increases
the permeability of various antibiotics (Snyder and McIntosh, 2000). More-
over, the outer membranes contain various porins that, although unspecific,
discriminate against molecules according to size, charge and hydrophobicity.
The inner membrane however is considered to be a highly specialized per-
meability barrier since it contains most of the functional transport systems.
Only a few nonpolar compounds can freely dissolve in the lipid bilayer and
cross the membrane without help, for polar and charged compounds or ions a
membrane protein is needed to pass the membrane barrier. These transporter
proteins function in different ways and can assist passive diffusion as well as
active transport against an electrochemical gradient. They can also be highly
specific for their substrate and be driven by ATP or by coupling with another
compound that transports down its electrochemical gradient. The so called
mechanosensitive channels respond to membrane tension. For example,
when the mechanosensitive MscL channel is placed into different lipid envi-
nvironments it is clear that an asymmetry of the lateral pressure in the bilayer is
required for MscL to open (Martinac, 2004).

Lipids by themselves and in mutual aid with transport proteins together
with LPS are the key to a functioning permeability barrier. The ability for
headgroups to form hydrogen bonds with other lipids or surrounding com-
pounds is believed to affect the permeability. In this respect glycolipids seem
less prone to hydrogen bond with other lipids than the phospholipids; never-
theless glyco-headgroups are also capable of forming hydrogen bonds be-
tween each other (Hinz et al., 1991). It has been shown that a mutant with
only one third of the normal amount of PE is hypersensitive to various anti-
biotics (Raetz and Foulds, 1977). This might be due to the lipid itself its
cooperation with transport proteins. Permeability is a phenomenon that is
jointly achieved by different lipids and proteins and is therefore a complex and tightly integrated phenomenon that is still only partly understood.

Cell division

The ability to divide is one of the most important features for prokaryotic cells, and it is also one of the most complex. In *E.coli* the cell division requires a minimum of 15 proteins that take part in constructing the so called constriction ring. These proteins localize in the cytosol, in the inner membrane; peripherally or membrane-spanning, and in the periplasm (Vicente et al., 2006). The process can be divided into at least three parts; recognition of division site, formation of the division ring and septal invagination and coordinated peptidoglycan synthesis which gives two daughter cells.

To direct the division site to the right place in the cell at least three Min proteins, MinD, MindE and MinC, are required in *E.coli*. The *minC* and *minD* gene products act jointly to form a nonspecific inhibitor of septation that is capable of blocking cell division at all potential division sites. MinC is the division inhibitor that blocks Z-ring formation *in vivo* and is capable of interfering with FtsZ polymerization *in vivo* (de Boer et al., 1992; Hu and Lutkenhaus, 2000) while MinD is an ATPase that is an activator of MinC function (Hu and Lutkenhaus, 2000; Huang et al., 1996). MinE acts in the opposite mode preventing the MinCD inhibitor complex from acting at central division sites while permitting it to block septation at polar sites (Huang et al., 1996). MinE proteins assemble in a ring that moves from the midcell to the poles in a wave-like fashion and stimulates the removal of MinD. In this way the concentration of division inhibitor is lowest at midcell (Hale et al., 2001; Hu et al., 2003). For proper function these proteins have to be membrane associated. MinC on its own is a cytoplasmic protein, but interacting with the peripheral MinD causes it to bind to the membrane (Hu and Lutkenhaus, 1999).

The division ring starts from the cytosol and moves outwards, recruiting membrane and periplasmic proteins (Vicente et al., 2006). The ring forming FtsZ assembles into protofilament sheets and minirings (Erickson et al., 1996). It is the most abundant of the known cell division proteins with as much as 10,000-20,000 copies per cell in a fast growing *E.coli* culture (Bi et al., 1991). This is also the protein that acts prior to the other septum forming proteins (Begg and Donachie, 1985). FtsZ as well as FtsA, ZipA and ZapA take part in the assembly of the division proteins. FtsA and ZipA connect the so called Z ring to the membrane and FtsK coordinates the septation with chromosome separation. FtsI and FtsW take part in synthesizing new peptidoglycan cell wall and AmiC and EnvC hydrolyses the cell wall thereby allowing the final separation of the cell (Weiss, 2004). There are also a handful of proteins essential for the division ring but with unknown function. The
proteins involved in the early phases of division exist in the cytosol, but are connected by peripheral and transmembrane proteins to the membrane. The proteins involved in the late phases of division either span the membrane or are present in the periplasmic space (Vicente et al., 2006). Recall that in the AD93 mutant, lacking the major lipid PE, both MinD and FtsZ are misassembled (Mileykovskaya et al., 2003; Mileykovskaya et al., 1998) demonstrating the relevance of membrane lipids for the assembly of at least two early cell division proteins.

**Osmotic stress tolerance**

In order to survive in various environments prokaryotic cells have the ability to withstand differences in external osmolarity. They respond to osmotic upshift in three overlapping phases: i) dehydration, ii) adjustment of cytoplasmic solvent composition and rehydration, and iii) cellular remodeling (Wood, 1999). When a wildtype *E. coli* cell is exposed to an external osmotic upshift the stress response is carried out by a number of membrane proteins. It is the influx of K⁺ by the Kup, Trk and Kdp systems (Kempf and Bremer, 1998), regulation of water by aquaporins such as AqpZ (Calamita, 2000), and accumulation of osmoprotectants such as glycine betaine and proline by OmpC, OmpF, ProU, ProP and BetP (Kempf and Bremer, 1998). Together these compounds serve to increase the free water content of the cells at high osmolarity, and maintain a positive turgor and thus permit continued cell proliferation.

Upon osmotic downshift (or hypo-osmotic stress) there are three contributing and identified *E. coli* mechanosensitive channels, the MscL, MscS and MscK (Levina et al., 1999; Li et al., 2002; Sukharev et al., 1994). These almost lack ionic specificity which can be useful when wanting to compensate for cellular turgor. The aquaporins also participate in the tolerance of osmotic downshift (Kempf and Bremer, 1998). Microarray studies implicate 60 uncharacterized genes in the osmotic stress regulon, which could mean that many more proteins or new osmotic stress response systems are involved (Cheung et al., 2003).

It is now clear that the mechanisms by which these turgor regulating proteins adjust the osmotic upshift are different from the ones regulating osmotic downshift. The proteins involved in the osmotic upshift response are regulated by the concentration of luminal ionic osmolytes. This means that they are switched on when the water content inside the cells becomes too low and the ionic concentration inside of the cell increases. One important part of the regulation seems to be a charged C-terminal domain found in both BetP and ProP and which is believed to exist also in OpuA and might associate with the anionic lipid bilayer (Poolman et al., 2004). Additionally it has been shown for OpuA that the rate of uptake increases with increasing
amounts (to about 40 percent) of anionic lipid DOPG (van der Heide et al., 2001). The mechanosensitive channels regulating osmotic downshift on the other hand do not respond to the osmolytes as such but to mechanical forces inside of the membrane (Martinac, 2004). *E.coli* MscL shows a gating mechanism strongly dependent on lipids with different geometries (Perozo et al., 2002). The channel opens up when the membrane is put under stress i.e. under hypoosmotic stress (Betanzos et al., 2002);(Perozo et al., 2002). In general it seems like one main feature of the osmotic regulating transporters/channels is that they are highly dependent, although differently, on what sort of lipids they are surrounded by.

Transmembrane protein topology

When the nascent chain of a bacterial transmembrane (TM) protein leaves the SecYEG translocon channel laterally it has to decide in what direction it wants to insert into the membrane. Moreover most inner TM proteins contain several membrane spanning alpha helices that have to orient themselves; both in order to each other and in context to the membrane. The answer to how a TM protein orients seems to lie within the amino acid sequence of the protein itself.

One of the most established rules is the “positive inside rule” which states that the positive amino acids are more abundant in the cytosol than in the periplasm (von Heijne, 1986). This rule is valid for many different membranes in all types of organisms (Wallin and von Heijne, 1998). There is also a tendency for negatively charged amino acids towards the outside but this is not as strong a determinant as the positive amino acids (von Heijne, 1992). Interestingly, changing the flanking charges to the opposite charge can reverse the topology of a segment (Beltzer et al., 1991; Parks and Lamb, 1991). The length of the TM segment is also a topological determinant where longer sequences support the C-terminal part to stay in the cytosol (Wahlberg and Spiess, 1997). Furthermore one stable oriented TM segment can “push” another neighboring segment towards one direction or the other probably due to close interactions (Heinrich and Rapoport, 2003). Other determinants are the hydrophobicity of the TM sequence and glycosylation (Goder and Spiess, 2001). There are probably even more topology determinants throughout the sequence that have not yet been found.
Figure 8. The topology of lactose permease (LacY) in *E. coli* cells with normal amount of phosphatidylethanolamine (about 75%) to the left and without phosphatidylethanolamine to the right. The organisation of TM VII in PE lacking cells is still unknown (Bogdanov et al., 2002).

It has been shown that in the Endoplasmic Reticulum direct lipid-protein interaction is important for the recognition of TM helices by the translocon (Hessa et al., 2005). This could mean that lipids possibly influence the insertion and topology of a membrane spanning protein. For example constructs have been made of Leader Peptidase containing zero to four positively charged amino acids just after the second TM spanning segment towards the periplasmic side. Introducing these constructs to wild type as well as an *E. coli* lipid mutant, containing only anionic lipids in the membrane (recall AD93, lacking PE), showed that fewer positively charges were needed for the segment to remain on the cytosolic side. Importantly, it was also shown that these effects were not due to the function of the Sec-machinery (van Klompenburg et al., 1997). Studies on the effect of anionic lipids on translocation have also been carried out in liposomes, thus Sec-independent insertion. It was shown that several anionic lipids stimulated translocation of an almost uncharged segment (Ridder et al., 2001). As mentioned earlier three TM proteins have changed topology in the PE lacking strain AD93. Moreover, both PC and MGlcDG could correct the topology for LacY, meaning surface charge is important for correct insertion of this protein (Bogdanov et al., 2002; Xie et al., 2006).

Clearly, not only the amino acid sequence of a TM protein is important for its correct insertion but anionic lipids, at least for some segments and proteins, are also topology determinants. In addition it appears as if the Sec-machinery is dependent on the surrounding lipids.
Summary of papers

Paper I

For the first time alMGS synthesizing the nonbilayer prone lipid MGlcDG in *A. laidlawii* was cloned and the activity was determined *in vitro* as well as *in vivo*. The aims were to analyze at both sequence and protein levels the regulatory features recorded for the purified native enzyme. Related genes were found in diverse species, for example in both *S. pneumoniae* and *A. thaliana*. To show that these potentially homologous enzymes could have the same activity, the similar *S. pneumoniae* gene was chosen for cloning and activity studies. The activity was indeed the same as the alMGS producing the MGlcDG lipid. However, when comparing activation by different lipids these two enzymes showed quite different activation patterns. The most drastic difference was that alMGS was highly stimulated by both PG and CL but that spMGS was only stimulated by PG. One reason for this might be the difference in the theoretical pI of the N-terminal part of the enzymes. Both enzymes have a much higher theoretical pI for the N-terminal domain than for the C-terminal part, but the N-terminal domain of spMGS has a lower pI than the corresponding domain of alMGS. Interestingly the *in vivo* activity for spMGS in *E. coli* was much lower, almost nonexistent, whereas for alMGS almost 10 molar percent MGlcDG lipid of the total lipid amount was produced in wild-type *E.coli*.

By a 3D fold prediction method the alMGS was also similar to the *E. coli* MurG with determined structure. Two regions showed high similarity; a potential membrane associated domain and a UDP-sugar binding domain. From the similarity with MurG combined with the lipid activation pattern a model of the alMGS bound to the membrane was proposed (see fig. 5). This model, as MurG, is composed of one N-terminal and one C-terminal domain with a cleft in-between. The membrane association is proposed to be accomplished by the more positively charged N-terminal part, which also contains presumed amphipathic helixes, preferably towards anionic lipids. The catalytic domain is proposed to be situated in the cleft in-between the two domains. This proposal is supported by the fact that the EX7E- motif, proposed to bind UDP-Glc, is situated inside of the cleft.
In summary, both alMGS and spMGS enzymes belong to a large group of glycosyltransferases widely spread in nature. They are probably structurally similar to the *E. coli* MurG and attach to the membrane both by electrostatic and hydrophobic interactions. Their activation by lipids is on the other hand dissimilar and this might reflect a need for different regulation in their native membranes.

Paper II

In this paper the alDGS, producing bilayer prone lipid DGlcDG, was cloned and the *in vitro* activity was determined. The aims were to potentially find the sequence features of the enzyme, responsible for the curvature regulation. Also this gene had numerous uncharacterized homologous genes in diverse organisms, one of them in *S. pneumoniae*. The *S. pneumoniae* gene was therefore chosen for cloning and the *in vitro* activity was determined. As suggested the spDGS also produced DGDG lipid but in this case the enzyme used UDP-galactose as substrate and MGlcDG as acceptor molecule producing GalGlcDAG lipid. Similar to spMGS, the spDGS did not respond to CL but only to PG. The alDGS was on the other hand clearly enhanced by both CL and PG. Nonbilayer prone DOG did not enhance the activity of spDGS, but the activity of alDGS was stimulated by DOG. Certainly, these corresponding DGS enzymes, as the two corresponding MGS enzymes, are differently regulated by the tested lipids.

Interestingly, building structural models of alMGS and alDGS as well as the corresponding enzymes from *S. pneumoniae* showed a large structural similarity. Also the model of cucumber MGS (scMGS) was remarkably similar to the other MGS enzymes (see fig. 4). All these models are similar to *E. coli* MurG and to the suggested model in paper I with two domains and a cleft in-between. However, having a closer look at these structural models small differences are noticeable, especially when looking into the surface charge of the different enzymes. The surface charges of both *S. pneumoniae* enzymes are much more evenly distributed on the surface of the two domains compared to the *A. laidlawii* enzymes. Especially alMGS has a very strong patch of cationic residues in its N-terminal part. When comparing the theoretical pI:s of all five structural models for the N-terminal part, both *A. laidlawii* enzymes have higher pI values than the *S. pneumoniae* enzymes. Moreover the pI of the C-terminal parts are also much lower for the *S. pneumoniae* enzymes. This suggests that the surface charges of the corresponding enzymes are partially the answer to the dissimilarity in regulation by anionic lipids. The alDGS also has more hydrophobic sequence segments potentially interacting with the bilayer interface.

Constructs of GFP attached to whole, N-terminal and C-terminal alMGS were produced. Transforming these constructs into *E. coli* showed larger
membrane binding for the whole enzyme than for the N-terminal part. The C-terminal part however, ended up in the cytosol. This shows that aLMGS is membrane bound and that both domains take part in the binding but the N-terminus contributes to a larger extent to the membrane association of the enzyme.

In conclusion, aLMGS, aLDGS, spMGS, spDGS and scMGS belongs to the same large structural class of glycosyltransferases as MurG. The model of aLMGS binding with the membrane in paper I seems to fit reasonable well with the structural models. The A. laidlawii and the S. pneumoniae enzymes are differently regulated by lipids, probably partially due to that they show variation in surface charge patterns.

Paper III

Here we took a step from the enzymatic world and used the previously studied enzymes to look at the different roles of membrane lipids. By introducing aLMGS to the PE lacking strain AD93 we got this new strain to produce about 50 percent nonbilayer-prone MGlcDG lipid. This was the first time that E. coli had possessed this large amount of a foreign lipid. The idea was to replace PE with MGlcDG to see whether this nonbilayer-prone lipid could, for some membrane associated functions, cure the malfunctioning AD93 strain. We also verified that there were no intracellular membranes by staining with the lipid stain FM4-64.

The first obvious difference with this new MGlcDG strain was that it grew much better than the AD93 strain. When making growth curves with different concentrations of Mg^{2+}, one could clearly see that the MGlcDG strain was not only growing better but also demanded less Mg^{2+} for growth. This shows that this strain could compensate for PE for cell viability, indicated by a lower requirement of Mg^{2+}, which signifies a more proper membrane physical state. When studying cell division by measuring the cell length, there was also a clear difference. The MGlcDG cells are much shorter than the AD93 cells; even so they are still not as short as the PE plus cells. When studying the osmotic stress tolerance, which is an indicator of the state of numerous transporters as well as “cell fitness”, the MGlcDG lipids completely compensate for the loss of PE. The AD93 strain almost stops to grow at concentrations higher than about 0.4 M of either NaCl or KCl, while both the PE plus strain as well as MGlcDG strain grow quite well even at 0.75 M NaCl/KCl. Moreover, the MGlcDG strain is a prototroph as the wild type while the AD93 is an auxotroph, meaning that it can not produce all essential compounds in a minimal growth medium. For the first time it was also shown that MGlcDG lipid could partially replace the loss of PE for the activity of LacY, which is almost zero in the AD93 strain. On the other hand, when studying permeability of the mutants both by several anti-
biotics as well as release of RNase it was obvious that the PE plus strain was the one strain with outstanding permeability barrier. This indicates the importance of hydrogen bonding, possible by the PE lipid, for preserved permeability.

Summarizing the results, clearly showed that MGlcDG can compensate for the loss of PE, partially or totally, for several membrane associated functions. Now the question remains: What physical state of the MGlcDG lipid can compensate for PE? Is it the nonbilayer tendency, the neutrality, or the possibility to hydrogen bond? Since AD93 only has anionic lipids it might be that MGlcDG just normalizes the membrane surface charge and this is explaining why the strain is more viable. Talking against this is the lower requirement of Mg\(^{2+}\) for growth, since it is believed that the need for divalent cations of the AD93 strain comes from making the CL lipid more nonbilayer-prone. To investigate this we have to compare this new MGlcDG strain with lipid strains containing other neutral lipids, but which are bilayer prone. For this we should move on to paper number IV.

**Paper IV**

This is a follow up story of paper number III, where the first goal was to investigate what lipid properties that could replace neutral and nonbilayer-prone PE for different membrane-associated processes. We now introduced alDGS as well as *Arabidopsis* MGDG1 and DGDG2 to AD93 to produce new lipid mutants of *E. coli*. Also here *E. coli* got significant amounts of foreign lipids DGlcDG, MGalDG and DGalDG, respectively. Interestingly, the DGDG strains have more CL than the MGDG strains, which indicates that *E. coli* tries to compensate for the loss of nonbilayer prone PE or MGDG. However, in all other studies we got unexpected results. The DGalDG strain grows and is dependent on Mg\(^{2+}\) at the same level as the crippled AD93. The cells are also much longer and the permeability is increased compared to the other glyco-mutants. Unfortunately, it was impossible to compare all the glyco-mutants regarding osmotic stress tolerance since the mono- and di-galactoDAG amounts dropped severely in quite low amounts of NaCl/KCl, probably due to quenching of enzyme interface-binding.

For the studied membrane effects the DGalDG strain stood out compared to the MGlcDG, MGalDG and surprisingly also to the DGlcDG strain, which all three showed about the same phenotype. If the DGlcDG can compensate for the loss of PE why then can not the DGalDG do the same thing? Both DGlcDG and DGalDG are neutral and bilayer-prone lipids. By introducing two different pyrene probes to liposomes made from all the glyco-strains, AD93 and the PE plus strain, we wanted to see whether there were any membrane lateral pressure differences in-between the strains. These experi-
ments show that there is a lower pressure in the acyl chain region for both DGlcDG and DGalDG strain than the others but the pressure is lowest for the DGalDG strain. This indicates that DGalDG is more bilayer-prone than the DGlcDG lipid. Supporting this is the difference in head group area occupied by DGalDG and DGlcDG shown by Iwamoto and colleagues (Iwamoto et al., 1982) (see figure 9). Moreover, the DGalDG strain produces less MGDG and CL than the DGlcDG strain which might contribute to the change of the pressure profile. These features were supported by published NMR analyses of DGalDG headgroup structure. Hence, DGalDG may occupy a slightly larger lateral area, enough for bringing curvature to a critical value.

In conclusion, it is obvious that the MGalDG and the DGlcDG, as the MGlcDG (Paper III), can partially compensate for the loss of PE for several membrane associated functions, most likely by diluting the high anionic surface charge of AD93. However, the DGalDG lipid can not substitute for PE in any of the tested membrane processes. Even if both DGlcDG and DGalDG are bilayer forming lipids they must have some difference/differences that are crucial for the physical state of the E. coli membrane. Pyrene data shows that the DGalDG strain has a lateral pressure that is most changed compared to the wild type strain, and this change might be out of the acceptable range for the membrane of E. coli. Hence, curvature stress is an important factor also for E. coli lipid bilayer properties.

**Figure 9.** Molecular models of A) DGalDG from thylakoids and B) DGlcDG from A. laidlawii (Iwamoto et al., 1982).
Livet är fett! - populärvetenskaplig sammanfattning på svenska

(Swedish summary)

En människa består av hundra tusentals miljarder celler men det finns även små organismer som bara består av en enda cell. Runt var och en av dessa celler finns ett hölje av fett (= lipider) som skyddar insidan av cellen från dess omgivning. Detta hölje, som kallas membran, fungerar framförallt som ett skydd från omgivningen. En delämnen som cellen behöver måste kunna passera via små kanaler (= proteiner) som är ”inbäddade” i lipidlagret. Lipider består av ett huvud som är vattenlösligt och en svans som inte är vattenlöslig. Det är därför fördelaktigt för lipider att existera i två lager där huvuderna i båda riktningar når vatten samtidigt som svansarna stickar in mot varandra och skylar sig själva från det omgivande vattnet. Det finns en mängd olika typer av lipider med olika egenskaper, form och laddningar men det finns lite kunskap om varför det finns så många olika lipider och hur de påverkar olika celler och membranproteiner. Idag riktas de flesta mediciner mot membranproteiner, men med mer kunskap om lipidsyntes och hur de påverkar proteiner skulle vissa mediciner istället kunna riktas mot lipider. Därför har målet med denna avhandling varit att ta reda på mer om både hur lipider syntetiseras men även om vilken effekt som olika lipidegenskaper får på vissa membranrelaterade processer i cellen.

Sockерlipider

Membranlipider med sockergrupper som huvuden är de absolut vanligaste på jorden framförallt eftersom de finns i växters fotosyntetiserande membran. Sockerlipider finns även i vissa membran i människor, djur och i vissa typer av bakterier. Vi har bevisat att två besläktade enzymer (katalyserar en reaktion) producerar sockerlipider i två olika bakterier. En av dessa bakterier, Streptococcus pneumoniae, är en bakterie som orsakar lunginflammation hos människor och tillhör en av de vanligaste dödsorsakerna hos gamla och barn i U-länder. Vi har även kunnat visa att dessa enzym tillhörlor en stor grupp av enzym, med liknade 3-dimensionell struktur, som bildar sockerlipider alternativt andra molekyler med socker (artikel I och II). Dessutom har vi visat att de två studerade enzymerna regleras på olika sätt i membranet. Det beror troligtvis på att enzymen
kommer från olika miljöer och därför kan de respektive membranen ha helt skilda behov. Eftersom vi nu vet att de studerade enzymerna bildar sockerlipider kan man lättare hitta närbesläktade enzym som tillverkar liknande lipider i andra organismer.

Olika former på fett

De enzym vi studerade i artiklarna I och II bildar två olika typer av sockerlipider, nämligen med en respektive två sockergrupper som huvud. Lipider med två sockergrupper får ett större huvud än en lipid med endast en sockergrupp och de har därför olika form i membranet. Men kan säga att lipider med ett litet huvud är koniska medans lipider med ett större huvud är cylindriska. Lipider med ett mindre huvud, som inte bara är sockerlipider, finns i de allra flesta naturliga membran. I artikel III och IV ville vi veta om de koniskt formade lipiderna har någon påverkan på membranproteiner eller om det är andra egenskaper hos lipiderna som är viktiga.

För att kunna studera lipidegenskaper har vi använt oss av en mutant av tarmbakterien *E. coli* som saknar sin vanligaste lipid PE (fosfolipid, normalt 70-80 %), som både är neutral och koniskt formad. Denna *E. coli* stam har då enbart cylindriska samt negativt laddade lipider. Genom att införa gener för olika sockerlipid-producerande enzym har vi fått nya stammar av *E. coli* som istället för PE bildar stora mängder av sockerlipider som *E. coli* normalt saknar. Dessa, för *E. coli*, nya lipid är allt neutrala men både koniskt och cylindriskt formade. Genom att studera tillväxten och hur olika membran- associerade processer fungerar i de nya sockerlipid-stammarna har vi kunnat dra olika slutsatser på vilka lipidegenskaper som är viktiga. Vi har kunnat se att neutraliteten på lipid och även ett relativt litet huvud är viktigt för optimal funktion av vissa membranproteiner. Så slutsatsen blir att livet är inte bara fett- utan fett på olika sätt!
Acknowledgements

Nu vill jag passa på att tacka alla som bidragit till att den här boken kunnat skrivas och till alla er som gjort de här åren så roliga. Tack till alla på avdelningen för biokemi och biofysik för trevliga år och för hjälp med olika små och stora saker. Speciellt vill jag tacka:

Åke, min handledare. Framförallt för dina aldrig sinande idéer och din kärlek för forskning som smittat av sig! Tack också både till dig och Karin för riktigt goda middagar under årens lopp.

Professor Dowhan and team, my collaborators “over there”. Thank you so much for good collaboration through these years. Your lipid mutants were one big reason why I wanted to start as a PhD student. It will be really interesting to follow the advancements of our lipid mutant strains in the future!

Maria R, för att du fick mig att börja i Åkes grupp och för ditt jobb med YibD. För att du alltid funnits där för att diskutera saker med, jag menar vi var ju till och med gravid samtidigt, hur bra kan det bli! För att du alltid lyssnar och bryr dig om!

Tuulia, för att du är en så härlig människa och glädjespridare samtidigt som en kritisk forskare! Ingen annan har kunnat få mig att skratta så gott under de här åren!

Amélie, our terrific postdoc! Thank you for really good collaboration and help the latest years. Your effort with the galacto-strains made everything really beautiful in the end!

Hanna, vi som alltid går om varandra vilket är riktigt synd eftersom du är så trevlig! Det var otroligt tur att du tog över för mig när jag var mammaledig och äntligen fixade DGlcDG stammen.

Alex, especially for making the beautiful pyrene-probe studies but moreover for really fun laughs! It was really great (and hilarious) for us that you joined our team this latest year!
Maria E, Patrik S, Stefan B, Lu L, Susanna V, the “old” members of the group. You are all very nice and helpful people that introduced me, in a very good way, to the Membrane-group up in Umeå.

Lars Wieslander och Petra Björk, för tillgång och raktigt bra hjälp med fluorescens-mikroskopet. Det har alltid varit trevligt att komma bort till er!

Dan and Filippa, for really nice scientific discussions. Unfortunately none of the great things we have done together is shown in this thesis, but the word will soon be out there…!

Mikaela och Susanna, för trevligt samarbete. Tyvärr blev väl inte våra resultat så intressanta som vi trodde, men det var kul i alla fall och man vet aldrig vad som händer framöver!

Marika, det blev tråkigare på våning fyra när du försvann! Vi får helt enkelt bli bättre på att träffas hemmavid och ha fler trevliga middagar!

Karin, Hanna och Anna, mina Lundavänner som jag fortfarande har kvar även om jag lämnade er redan efter tre terminer. Tack för att ni finns och för att ni gjorde tiden i Lund så himla kul!

Karin HH, vi har ju faktiskt följt varandra först i Lund sen i Umeå och nu är vi båda här i Stockholm, fast det var i Umeå vi hittade varandra! Tack för många roliga stunder och för att du är en så härlig person och supervän!

Malin och Héléne, mina underbara Östersunds-vänner! Jag är så glad att ni fortfarande är mina bästa vänner och tack så otroligt mycket för allt stöd och alla skratt jag fått av er under åren!

Titti och Kent, mina gamla ☺ men härliga syskon för att ni alltid finns där för mig. Speciellt tack för att ni har gett mig mina fyra underbara syskonbarn Jenny, Frida, Daniel och Marie!

Mamma och Pappa, det går inte att beskriva i ord hur underbart härliga ni är, hur ni alltid ställt upp för mig eller hur mycket jag älskar er! Tack vare er är jag den obotliga optimist jag är, ni har gjort mitt liv underbart!

Valdemar, jag kunde väl aldrig tro att jag skulle få en sån underbar son som du! Tack för att varje stund med dig är ren lycka! Du är mammas hjärtegull!

Johan, tack för att du är världens kärleksfullaste pojkvän och pappa! Tack för att du alltid finns där och för att du alltid lyssnar på mig. ”Jag är alltid tryggast när du är en liten bit ifrån, en rörelse i ögonvrån…”


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