1.04 Microbial Polysaccharide Structures

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1.04.1 Introduction: Bacterial Polysaccharides

Bacteria possess a cell envelope which is a highly complex structure with a number of functions which may be separative (e.g., separation from the environment, protection from harmful influences) or connecting (e.g., transport of substances inside-out/outside-in, communication with the environment).[1] Bacterial cell envelopes provide the bacteria with sufficient rigidity and enable metabolism, growth, and multiplication. In general, bacterial cell envelopes allow for all of these functions; however, different bacteria involve different cell envelope molecules and architectures to be optimally operable. Thus, bacterial cell envelopes are complex and vary in a number of details. Based on overall architecture, the general classification distinguishes between Gram-negative, Gram-positive, mycobacterial and archaebacterial cell envelopes which does not reflect a variety of structural details.

In addition to protein and lipid components, bacterial cell envelopes contain a variety of glycans, which are classified as polysaccharides, lipoglycans, and peptidoglycans. This overview summarizes general features and recently analyzed chemical structures of the latter three groups. A number of reviews have been published which summarize earlier findings, some of which are mentioned here.[2–13]

1.04.2 Lipopolysaccharides (Endotoxins)

1.04.2.1 Introduction

According to the behavior in the Gram-stain, the domain of bacteria is divided into Gram-positive and Gram-negative bacteria and the outcome of this staining procedure is based on the cell wall architecture.[14,15] The cell walls of Gram-negative and Gram-positive bacteria differ fundamentally (**Figure 1**), and only the former contain an additional membrane, the outer membrane (OM), thereby creating an additional compartment, the periplasmic space. The outer membrane of Gram-negative bacteria is asymmetric with respect to the distribution of lipids whereby the outer leaflet is made from a phosphoglycolipid called lipopolysaccharide (LPS, endotoxin), while the inner leaflet is made from phospholipids.[16] The number of LPS molecules per cell in Gram-negative organisms has been estimated to $\sim 2 \times 10^6$ molecules.[17]

The genera *Escherichia*, *Salmonella*, *Klebsiella*, *Proteus*, *Yersinia*, and *Shigella*, among others, make up the family of Enterobacteriaceae and thus are members of the harmless or even vitally important commensal flora of mammals. However, the same genera also comprise important pathogens which are able to cause infections. Infection may occur by invasive bacteria, enterobacterial (e.g., *Escherichia coli*, *Salmonella enterica*, *Shigella*) and nonenterobacterial (e.g., *Neisseria*), which are able to penetrate the mucosa and the endothelium and may reach subepithelial tissues and the bloodstream. Also following traumatic stress, surgery, and, for example, severe burns, the protective barriers are broken and leakage of bacteria may occur, whereby LPS and other microbial products reach the blood circulation. Primary immune recognition by dendritic cells, neutrophils, and macrophages lead to the activation of innate immune responses which have developed to identify and, if possible, eliminate the potentially life-threatening microbes from the infected site. Recognized target molecules comprise LPS and other microbial products which are commonly referred to as pathogen-associated molecular patterns (PAMPs). Various receptors of immune cells are involved in the innate immune recognition (pattern recognition receptors, PRRs), among which Toll-like receptors (TLRs) have been shown to play a prominent role and specifically recognize molecules belonging to PAMP.[18,19] Apart from the innate defense system, the adaptive immune response is also activated in mammals. By orders of magnitude the most potent

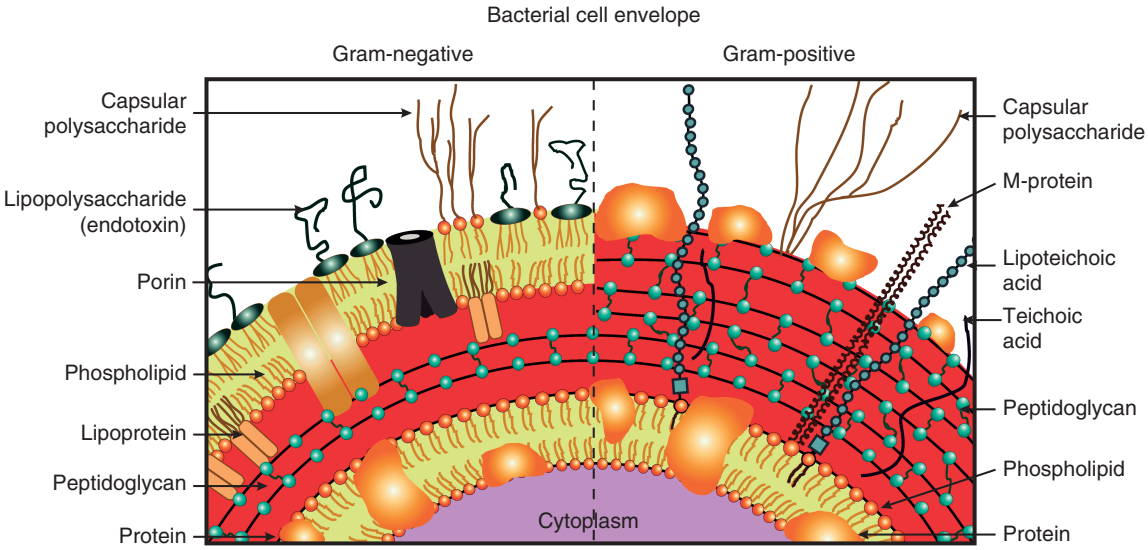

Figure 1 Schematic representation of the membrane organization of Gram-positive and Gram-negative bacteria. Only Gram-negative bacteria contain an outer membrane (OM) creating an additional compartment, the periplasmic space. The outer layer of the OM is composed of lipopolysaccharides (LPSs) whereas the inner layer is composed of phospholipids.

stimulus of all PAMPs is LPS of a characteristic chemical structure exemplified by LPS from *S. enterica* and *E. coli*.[20] Biophysical studies have revealed that LPSs adopt certain physical aggregate structures and a conical shape of the individual molecules is associated with biological activity.[21]

The activation of the immune system may lead to the eradication of the infectious bacteria at the infected site; however, dissemination of bacteria may occur accompanied by an initial overwhelming hyper inflammatory response (systemic inflammatory response syndrome, SIRS), which is counter-regulated leading to suppression of the immune system and the failure to accurately manage the infection.[22] This scenario may lead to organ failure and death, a complication known as septic shock. Septic episodes, with an incidence of estimated >750000 cases each year in the United States of America, are associated with a high mortality rate among patients who develop septic shock, ranging in severe cases from 30% to 70%.[23] Apart from Gram-negative bacteria, sepsis can be elicited also by other pathogens such as Gram-positive bacteria, viruses, and fungi.

Already in 1892, Richard Pfeiffer attributed toxic effects to the action of heat-stable components of the Gram-negative bacterium *Vibrio cholerae*. The term endotoxin was thus introduced to distinguish this class of toxins from actively secreted heat-labile exotoxins, and subsequently LPSs were recognized as endotoxins (see below). Reviews on the history of endotoxin have been published recently.[24,25] Despite the fact that many of the molecular events during the activation of the immune system by LPS have been elucidated in *in vitro* test systems[18,23,25] and animal models of septic shock,[26] and despite the availability of improved antibiotic treatment even today, the treatment of septic shock is still difficult.[22,25]

The amphiphilic nature of LPS leading to larger aggregates in solution and difficulties in obtaining pure preparations precluded a detailed structural analysis for decades and therefore an establishment of structure–activity relationships. Only after the development of extraction procedures for the preparation of homogeneous LPS,[27,28] chemical and biological meaningful experiments could be performed. For the structural analysis, the application of degradation by chemical means was necessary and it was realized that upon treatment with mild acid in water, a precipitate could be obtained which was termed lipid A and which represented the lipid anchor of the LPS molecule.[29] Its exact chemical structure (see below) remained elusive for several decades due to the difficult chemistry of this complex molecule. In 1983 the structure was elucidated and unequivocally proved to be correct after its chemical synthesis.[30] The available material also paved the way for the identification of lipid A as the endotoxic principle of LPS[31–33] and the acquired knowledge allowed then the detailed investigation of structure–activity relationships,[34–36] including a biophysical characterization of biologically active and inactive lipid A.[37]

Although biological activities of the isolated LPS have been well established *in vitro* and *in vivo*, there was some uncertainty with regard to the role as virulence factor during natural infections. The improved knowledge of the biosynthesis of lipid A[38] together with the established structure–activity relationships, allowed the construction of mutant bacteria which express functional but nontoxic lipid A[39] which showed that upon infection of mice despite an *in vivo* growth rate comparable to the wild-type strain, the mutant was unable to cause disease. LPS can thus be regarded an important virulence factor also during infections with Gram-negative bacteria. It is therefore expected that a detailed chemical characterization of bioactive LPS and the establishment of structure–function relationships in terms of immune cell activation will allow an antiendotoxic treatment during infections with Gram-negative bacteria and possibly help to prevent the development of septic shock. Since LPS is essential for the viability of most Gram-negative bacteria (discussed below), critical enzymatic steps for its biosynthesis represent attractive targets for the development of novel antibiotics[40] and inhibitors of lipid A and Kdo biosynthesis have been developed.[41,42] A detailed knowledge of structure–function relationships of LPS in bacterial membranes may also lead to novel antiinfectious agents. Since LPS are surface molecules which are frequently accessible to antibodies, they represent potential targets for vaccination.

1.04.2.2 Functional Aspects of LPS

In bacteria which are not encapsulated, LPSs are exposed on the surface of the bacterial cell and thus represent the first-line defense against various chemical and physical stresses associated with the natural habitats of bacteria. In particular, in situations where an infection in mammals is established, some pathogenic bacteria are able to resist the mounted counterattack consisting of a whole range of antimicrobials in activated serum, for example, antibacterial peptides, antibodies, the deposition of complement and formation of the membrane attack complex, in addition to ingestion and destruction by cellular phagocytosis. Some Gram-negative bacteria are able to evade the serum attack by their ability to grow intracellulary within mammalian cells (e.g., *Chlamydia*, *Yersinia*, *Salmonella*, *Brucella abortus*). A similar ecological niche is occupied by nitrogen-fixing symbiotic bacteria such as *Rhizobium* in plants which has been

suggested to involve similar protective mechanisms,[43] and the chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* has been shown to depend on the structure of its LPS.[44]

LPSs have evolved to support bacterial growth in these very different environments. The environmental conditions may change accidentally or such changes are regular events as part of the biology of the bacteria, for example, the change of host species from rodent to insect and mammal by *Yersinia pestis*.[45–48] Bacteria have thus developed the ability to sense environmental changes in pH, salt concentration, and temperature by two-component regulatory systems such as PhoP/PhoQ and PmrA/PmrB. Such systems are also involved in the structural modification of LPS.[49–55] Random phase variation may also occur in a bacterial culture (e.g., in *Neisseria* and *Haemophilus*) which is based on regular genetic events[56] and leads to a preadaptation of a certain percentage of the culture to likely encountered environmental conditions.

The fact that LPSs have been preserved during evolution indicates their biological importance for the survival of Gram-negative bacteria. The outer membrane of these bacteria represents a permeation barrier which very effectively prevents the lateral diffusion of hydrophobic molecules such as detergents, bile salts, antibiotics, and large glycopeptides.[57–59] This is attributed to tight lateral interactions between a large number of anionic groups present in LPS molecules which are bridged by divalent cations such as Mg^{2+} and Ca^{2+}. These charged groups are mainly located close to the surface of the membrane. The fatty acids in LPS are highly ordered in a gel-like state and in a nearly crystalline arrangement with transition temperatures up to about 60 °C.[59] The unsubstituted hydroxyl groups of β-hydroxylated fatty acids have been suggested to participate in intermolecular hydrogen bonds, further strengthening the ordered structure of LPS in membranes.[59]

Due to the barrier properties of the outer membrane, Gram-negative bacteria have developed transport mechanisms to allow uptake of nutrients and export of waste products. They are able to use fatty acids as energy source, and their transport through the outer membrane is mediated by outer membrane proteins.[60] A recently solved crystal structure of the FadL protein from *E. coli*, which is involved in long-chain fatty acid uptake, gives an indication of how this transport is achieved.[61] Transport of hydrophilic substrates across the outer membrane is primarily mediated by passive diffusion through nonspecific or substrate-specific porins.[59] An exception is the energy-driven active transport of siderophores across the outer membrane.[59] LPSs form the matrix for those proteins which are embedded in the outer membrane and have been shown to be important for their correct folding,[62,63] oligomerization,[64,65] and functioning.[66,67] Therefore, mutations which lead to severely truncated LPS are known to affect the formation of a functional outer membrane referred to as the deep-rough phenotype which is characterized by a higher permeability toward hydrophobic agents.[57,59,68,69] Mutations which affect the available amount of functional LPS molecules in a cell are accompanied by the loss of the barrier function and lead to a hypersensitivity against hydrophobic substances[59] which has been explained by the introduction of patches of phospholipids into the outer leaflet of the outer membrane in order to compensate the reduced amount of available LPS.

Mutations in genes which are involved in early LPS biosynthesis are known to interfere also with the assembly of flagella and pili.[70–75] Structural analysis of the outer membrane protein FhuA which belongs to a family of proteins that mediates the active transport of siderophores has revealed by accidental co-crystallization a crystal structure of *E. coli* K-12 LPS and details of the interaction with this outer membrane protein.[76] In this complex most of the important hydrogen-bonding or electrostatic interactions with LPS were provided by eight positively charged residues of FhuA. A database search based on this complex has identified a similar structural motif of a subset of four amino acids in various proteins which are known to bind to lipid A and of which some are involved in innate immune responses.[77]

Cationic antimicrobial peptides (CAMPs) and proteins, like defensins and polymyxins, among others, effectively disintegrate the LPS assembly by targeting the negatively charged groups, and compromise the barrier function.[57,59,78] When grown in the presence of such antibacterials, *S. enterica* bacteria were isolated which showed a resistant phenotype which is correlated with the expression of structurally modified LPS (see below).[79] Some bacteria, like *Pseudomonas aeruginosa* and *Burkholderia cepacia* are known to be intrinsically resistant toward CAMP and have been shown to contain similarly modified LPS[80] (and references cited therein).

1.04.2.3 The Chemical Structure of Smooth(S)-Type LPS

LPSs are phosphorylated glycolipids which possess a complex chemical structure and many reviews on LPS structures and their biosynthesis has been published, some of which appeared more recently.[38,40,80–85] The lipid anchor of LPS, called lipid A, in most bacteria studied consists of an N- and O-acylated (β1→6)-linked D-glucosamine (GlcN)

disaccharide which is phosphorylated in positions 1 and 4′ (lipid A backbone). β-Hydroxylated fatty acids are characteristic components of lipid A which, for biosynthetic reasons,[38] always quantitatively substitute the 2- and 2′-positions of the backbone in an amide linkage. They may be further located at the 3- and 3′-positions in the ester-linkage and esterified at the β-hydroxyl group (secondary fatty acids).[80] Attached to the 6′-position of the lipid A backbone is a heteropolysaccharide of varying length via a ketosidic linkage involving either D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) in *Acinetobacter* or 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) in all other bacteria (for structural differences, see **Figure 2**).[83] In *B. cepacia,* Ko replaces the terminal Kdo residue[86] and in *Y. pestis* either a single Kdo or an α-Ko-(2→4)-α-Kdo-disaccharide is expressed.[87,88] The ketosidic linkage between the core and the lipid A is generally labile toward mild acid; however, replacement of Kdo by Ko leads to an increased acid stability of this linkage. Thus, mild acid treatment is often applied to cleave the lipid A from the remaining saccharides. Alternatively, strong alkaline hydrolysis may be applied for complete deacylation (see below).

In many bacteria, a core-oligosaccharide (core-OS), which, based on genetic and structural differences, may be further subdivided into an inner and outer core, connects a long carbohydrate chain, the O-polysaccharide (O-PS), to the lipid A. Within enterobacteria, a characteristic component of the inner core region is heptose which in *E. coli* and *Salmonella* mostly possesses the L-*glycero*-D-*manno* configuration (L,D-Hep, **Figure 2**); D,D-Hep has been found in LPS of *E. coli*[89] and bacteria other than *E. coli* and *Salmonella*.[82,83] However, after many structures have been elucidated, it becomes evident that a clear distinction between an inner and an outer core based solely on composition cannot be applied easily to LPS from many other bacterial species. Although Kdo or Ko have so far always been found in LPS, connecting the core with the lipid A, some LPSs do not contain heptoses.[83] In addition, heptose has been identified as component of the outer core (e.g., in *E. coli* K-12 and *Klebsiella*) and even the O-PS and, as we know now, the same holds true for Kdo. Typical examples of LPSs in which the core region can be divided into an inner and an outer core are LPSs from *Enterobacteriaceae* and *Pseudomonadaceae*.

In general, LPSs which contain an O-PS are referred to as smooth (S)-type LPSs due to a smooth colony appearance of these bacteria. Such bacteria express a mixture of LPS molecules which differ in the length of the O-PS leading to a characteristic banding pattern in polyacrylamide electrophoresis. Additional heterogeneity results from molecules devoid of the O-PS, called rough (R)-type LPS, and from nonstoichiometrical structural variations in all parts of the LPS molecule including the lipid A. R-type LPS can be isolated in large amounts from mutant bacteria which have a defect in LPS biosynthesis. Due to the low proportion of R-type LPS in wild-type bacteria, the first chemical analysis of core-OS was only possible from LPS of such mutant bacteria and phage typing was often used as a helpful tool.[90–97] Apart from these mutants, in certain other nonenteric pathogenic bacteria such as *Neisseria*, *Haemophilus*, *Chlamydia*, and *Y. pestis* only R-type LPSs are present which are naturally devoid of O-PS and contain short oligosaccharide chains instead.[82] The term lipooligosaccharides (LOSs) was introduced by some authors to distinguish

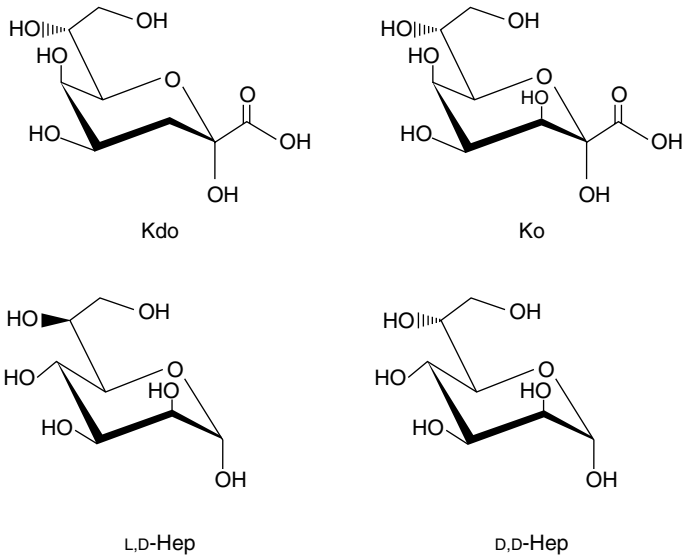

Figure 2 Chemical structures of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko), L-*glycero*-D-*manno*-heptose (L,D-Hep), and D-*glycero*-D-*manno*-heptose (D,D-Hep).

lipid A-bound oligosaccharides of *Neisseria* and *Haemophilus* which contain structures also found in mammalian glycolipids from other LPS.[98] The description of these chemical structures has been placed in Section 1.04.2.4.3.

Despite tremendous advances in both, knowledge and technology, the chemical analysis of LPS is even nowadays a difficult task due to the amphiphilic nature of LPS and the heterogeneity of isolated LPS preparations. Sophisticated techniques such as modern high-field nuclear magnetic resonance (NMR) spectroscopy and modern mass spectrometry in addition to conventional analytical chemistry usually have to be applied. The strong biological activity of certain LPSs requires the identification of even minor components in these preparations to fully understand biological effects. Furthermore, LPS preparations of highest purity are needed which seemed to be impossible for this class of glycolipids. Until recently, it seemed impossible to separate natural LPS into homogeneous fractions. However, the availability of a defined mutant together with improved knowledge of the biochemical and biophysical properties of this molecule in combination with sophisticated structural analysis seems to open the door to reach this goal in the future (see below).[99] Due to the enormous number of chemical structures elucidated to date, most of which have been subject to recent reviews, we will restrict our presentation to a few examples exemplifying general principles and would kindly refer the interested reader to the reviews published earlier, some of which we have mentioned here, or the original literature.

1.04.2.3.1 The lipid A

1.04.2.3.1.1 Chemical structures

Several detailed and excellent reviews of the chemical structures of lipid A and their biosynthesis have been published recently.[38,40,80,84] A lipid A structure which is widely distributed in Nature has first been elucidated in LPS from *Salmonella* and *E. coli* (**Figure 3**). It consists of a 1,4′-diphosphorylated (β1→6)-linked GlcN-disaccharide (GlcN I-GlcN II, lipid A backbone) which is substituted by *R*-3-hydroxymyristic acid [14:0(3-OH)] residues in positions 2, 3, 2′, and 3′ (primary fatty acids). 3-(or β)hydroxylated fatty acids identified so far always possessed the *R*-configuration and possess a chain length ranging from 10, as found in *P. aeruginosa*,[100] up to 22 carbon atoms as

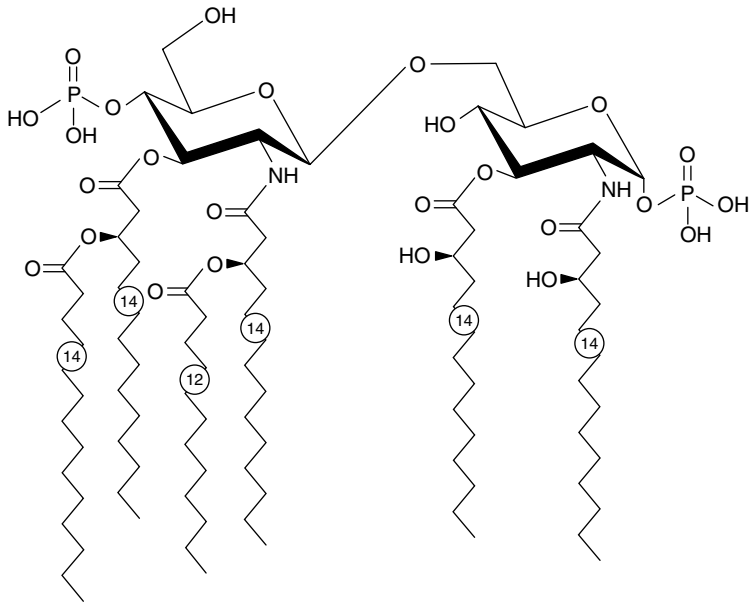

Figure 3 Chemical structure of the main lipid A molecule from *E. coli* and *S. enterica*. Depending on growth conditions structural variants are found which contain in the 1- or 4′-position 4-amino-4-deoxy-β-L-arabinose (Ara4N) or 2-aminoethanol phosphate (PEtN) connected to the 1- and 4′-monophosphates (for details see text). A diphosphate may be present in the 1-position instead of a monophosphate. Palmitate (C16:0) may be present as secondary fatty acid attached to the 3-hydroxymyristic acid [14:0(3-OH)] in position 2. The secondary myristic acid (14:0) may be replaced by *S*-2-hydroxymyristic acid [14:0(2-OH)]. When grown at lower temperature a 16:1 fatty acid replaces the secondary 12:0 at position 2′ in *E. coli*.

present in *Chlamydia*.[101–104] In Rhizobia,[105,106] *Agrobacterium*,[106] and *Legionella pneumophila*,[107] unusually long fatty acids of 28 C-atoms are present which are double the length of the fatty acids in lipid A from other bacteria and presumably span the whole lipid bilayer. In *E. coli* and *Salmonella* secondary acyl groups substitute the hydroxyl group of 14:0(3-OH) in positions 2′ (tetradecanoic acid, 14:0) and 3′ (dodecanoic acid, 12:0).

This type of lipid A is as well the main species found in other enterobacterial and nonenterobacterial genera and has been shown to exert the highest endotoxic activity *in vitro*.[20] In *S. enterica*, a heptaacylated lipid A species is synthesized in nonstoichiometric amounts which carries hexadecanoic acid (16:0) as secondary fatty acid at position 2 of GlcN I. In several lipid A from diverse species, 2-hydroxy fatty acids have been identified, including *Salmonella*, *Pseudomonas*, *Klebsiella*, *Bordetella*, and *Legionella*. These always possess the *S*-configuration.[108,109] So far, these have neither been found in primary positions nor further esterified. The degree of 2-hydroxylation appeared to be regulated by environmental factors such as growth temperature and structural analysis suggested that α-hydroxylation takes place at the fully acylated lipid A.[110] The biosynthetic pathway of lipid A in *E. coli* and *Salmonella* has been elucidated,[38] and it could be shown that the nonstoichiometric substitution with 2-hydroxymyristate [14:0(2-OH)] instead of myristate as secondary fatty acid in the 3′-position in *S. enterica* sv. Typhimurium is due to a hydroxylation reaction carried out by the LpxO enzyme.[111] This enzyme is missing in *E. coli*, but homologs have been identified in the above-mentioned bacteria which contain 2-hydroxy fatty acids.[84] Further structural modifications of lipid A in *Salmonella*, such as the addition of 16:0 and the removal of the 14:0(3-OH) fatty acid at the 3-position of GlcN I, are the result of the PagP[112,113] and PagL[84,114] enzymes, respectively, which are both located in the outer membrane. Homologs of the PagL enzyme have been identified in a variety of other genera, including *Bordetella*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, and *Azotobacter*.[115]

The distribution of secondary fatty acids, which are usually attached after the biosynthesis of the core region has been initiated,[38] determines the symmetry of the molecule. Lipid A containing a disaccharide as hydrophilic backbone may carry three to seven fatty acids which, if present in an even number, may be either symmetrically or asymmetrically distributed over the disaccharide backbone.[67] Examples of symmetrical lipid A structures (**Figure 4**) are found in *Chromobacterium violaceum*, *Rhodocyclus gelatinosus*, and *N. meningitidis*.

C. violaceum, as a typical representative, possesses a hexaacylated lipid A, which is composed of a 1,4′-diphosphorylated (β1→6)-linked GlcN-disaccharide as found in *E. coli* and which is substituted in positions 2 and 2′ with 12:0 (3-OH) and 3 and 3′ with 10:0(3-OH) as primary fatty acids. Secondary 12:0 are present in positions 2 and 2′. The 12:0 in position 2′ is nonstoichiometrically α-hydroxylated [12:0(2-OH)], leading to microheterogeneity. In some bacteria amide-linked fatty acids may be 3-keto-fatty acids as observed in LPS of bacteria which phylogenetically belong to the γ-3 subgroup such as *Rhodobacter capsulatus*, *R. sphaeroides*, *Paracoccus denitrificans*, and *Vibrio anguillarum*.[116] Unsaturated fatty acids, which are otherwise rarely found in LPS, are also present in lipid A from *R. sphaeroides*, *R. capsulatus* and *P. denitrificans*, *Y. pestis*, and in lipid A of *E. coli* and *Salmonella* grown at low temperature.[80,88,117–120]

Recently, the structural investigation of lipid A of the spirochaete *Leptospira interrogans* (**Figure 5**) revealed that its lipid A completely lacked negative charges and contains unsaturated fatty acids. While a diphosphorylated lipid A is synthesized first, the 4′-phosphate is removed at a later stage and the 1-phosphate is methylated.[121,122] Also in other bacteria, the lipid A backbone is frequently subject to structural modifications which comprise the addition at either or both phosphates of additional organic phosphate, 2-aminoethanol (EtN), 2-aminoethanol phosphate (PEtN), 2-aminoethanol diphosphate (PPEtN), GlcN, GalN, GalA, 4-amino-4-deoxy-β-L-arabinopyranose (Ara4N), D-arabinofuranose (Ara*f*), or the enzymatic removal of phosphate by phosphatases. Several of these modifications have been shown to be under the control of the two-component regulatory systems PhoP/PhoQ and PmrA/PmrB which are connected to each other.[123,124] Therefore, the structural modifications of LPS can be induced by low external Mg^{2+} concentration and low pH, mimicking the situation encountered in RAW 264.7 cells.[125] Groisman *et al.* have thus suggested that LPS serves as a reservoir for divalent cations which under limiting concentrations of Mg^{2+} and Ca^{2+} are mobilized by reducing the net negative charge.[126]

Since the same structural modifications have been connected to an increased resistance toward CAMP, they apparently also represent a natural defense mechanism. There is a great body of evidence showing that modifications which reduce the net negative charge of LPS are related to resistance toward polymyxins.[49,59,78,79,127–133] Inherently, polymyxin B-resistant bacteria such as *P. aeruginosa*, *Proteus mirabilis*, *C. violaceum*, and *B. cepacia* contain Ara4N almost stoichiometrically in their lipid A.[80] The incorporation of 16:0 by PagP which is also under PhoP/PhoQ control has been identified in polymyxin B-resistant mutants.[130] Therefore, it appears that apart from structural changes in the lipid A backbone, the acylation state influences polymyxin resistance also. Since Tran *et al.* have shown that the acylation state influences the addition of Ara4N to the lipid A backbone, this may be an indirect effect.[134] These structural modifications are accompanied by an increased bacterial cross-resistance toward other CAMP and proteins

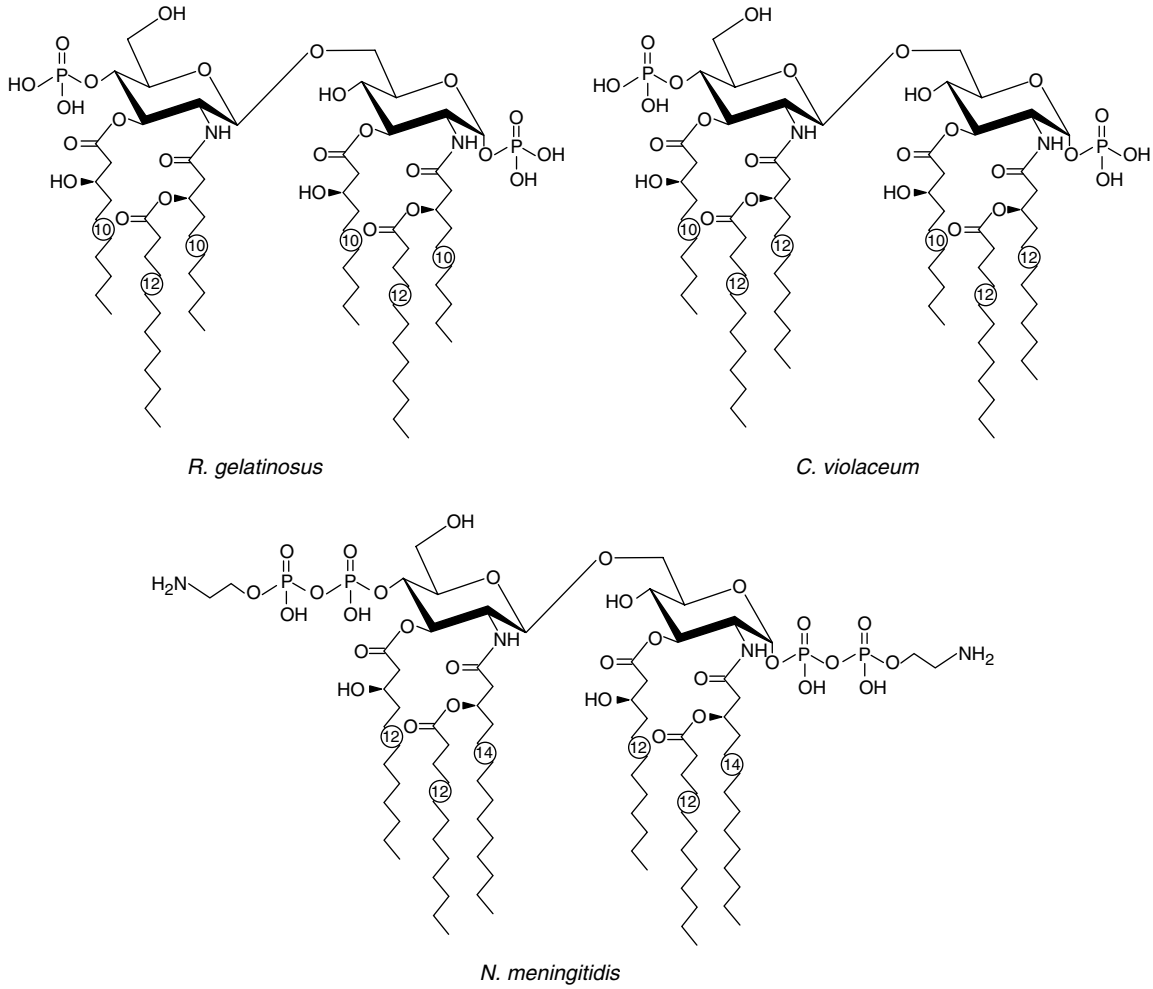

Figure 4 Lipid A from *C. violaceum*, *Rhodocyclus gelatinosus*, and *N. meningitidis* as examples of symmetrical lipid A.

in vitro and may also promote bacterial survival at infected sites in mammals, inside human neutrophils and macrophages.[52,125,135,136] However, the recent investigation of a *Y. pestis* mutant with a defect in late acyltransferases which is unable to generate hexaacylated lipid A was not more sensitive to polymyxin than the wild type.[137] This may not be surprising in the light of results obtained by Knirel *et al.* and others,[88,138] who investigated temperature-dependent changes in lipid A of *Y. pestis* biovars, and who showed that tetraacylated lipid A represents the main lipid A species under normal growth conditions. Also, the identification of a PmrA-regulated gene locus (*cptA*) required for the PEtN modification of the inner core did not result in an increased polymyxin resistance which implied that factors other than surface charge contribute to the resistant phenotype. A recent investigation of polymyxin resistance of *P. aeruginosa* also indicated that in this bacterium other reasons unrelated to lipid A modification may contribute to the observed natural polymyxin resistance,[139] and polyamines have been suggested to play a role.[140]

Incomplete acylation due to mutations of biosynthetic genes involved in the early steps of lipid A biosynthesis[38] (lpxA, lpxC, lpxD) leads to a phenotype of an increased hypersensitivity against hydrophobic agents.[58,141] Mutations which affect the attachment of secondary fatty acids and thus result in the production of tetraacylated lipid A species lead to an impaired growth on solid media and such bacteria are not viable when cultured in broth.[141] Helander *et al.* reported that in a permeability mutant (*ssc*) of *S. enterica* sv. Typhimurium, the predominant lipid A is heptaacylated containing additional 16:0.[142] Thus, it appears that apart from under acylation also additional fatty acids incorporated by PagP may compromise the barrier function of the outer membrane.

A detailed investigation of lipid A from *S. enterica* sv. Typhimurium revealed that the monophosphates of the lipid A backbone are partially substituted with two Ara4N in both, 1- and 4′-position, or as a single substitution in

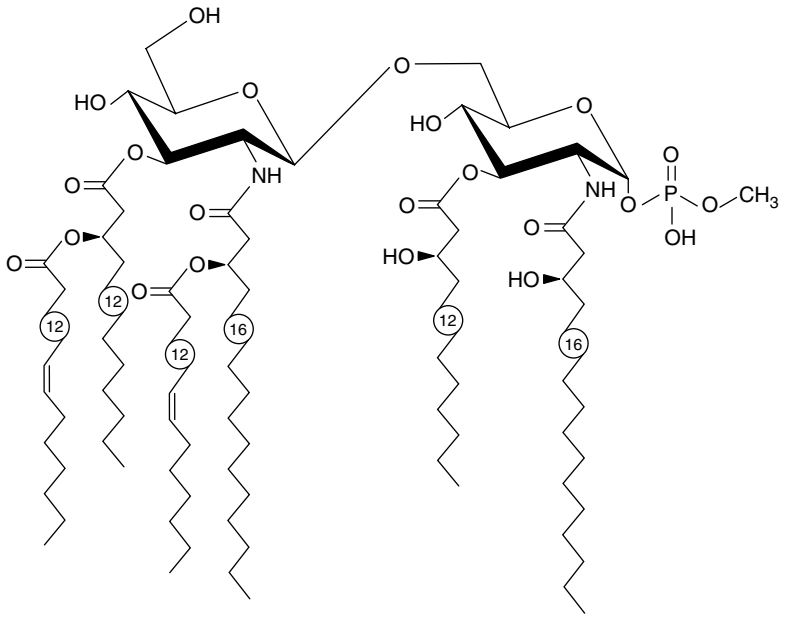

Figure 5 Lipid A of the spirochaete *Leptospira interrogans* completely lacks negative charges and contains unsaturated fatty acids. The Δ-12:1 is partly replaced by Δ-14:1 at both positions.

position 4′.[143,144] However, mutants which are defective in Kdo biosynthesis express a lipid A with Ara4N exclusively at the anomeric phosphate. This was explained by Zhou *et al.* by a dependence on the presence of Kdo of the ArnT enzyme,[144] which transfers Ara4N to the 4′-position. The Ara4N substitution may be alternatively replaced by PEtN. Although not seen in *E. coli* K-12 when grown under normal conditions, the same modifications have also been found after induction by metavanadate.[143,145] Surprisingly, no mixed substitutions have been identified, that is, Ara4N and PEtN in the same molecule, which may indicate that they mutually exclude each other. However, they may also be only minor components which are difficult to obtain in quantities sufficient for chemical analysis.

The lipid A from *Rhizobium leguminosarum* and *R. etli* totally lacks phosphate (**Figure 6**). Instead, GlcN I is converted into 2-aminogluconate and the 4′-phosphate is replaced by a galacturonic acid residue after enzymatic cleavage of this phosphate by a specific phosphatase. Thereby, the number of negative charges is retained and phosphate groups are functionally replaced by carboxyl groups. Additional lipid A components have been shown to contain also unmodified GlcN I and several structural variants have been isolated and chemically characterized. The complete biosynthetic pathway of this unusual lipid A structure has been elucidated.[146–153]

The presence of PEtN at the anomeric position of *H. pylori* lipid A, which has not been seen previously in other bacteria, where a PPEtN is always found instead, prompted Tran *et al.* to search for LPS modifying enzymes. They were able to identify a phosphatase activity which removes the anomeric phosphate of the lipid A which is replaced by PEtN in a second enzymatic step.[154] Thus, after the generation of an *E. coli* type lipid A, which is exported to the periplasmic face of the inner membrane, the LPS structure is further trimmed and modified. The removal of the anomeric phosphate is a prerequisite for further subsequent modifications in which the terminal Kdo, the 4′-phosphate, and a fatty acid are presumably enzymatically released.[155]

Studies aiming at the molecular mechanisms underlying polymyxin resistance in *P. aeruginosa* revealed structural differences in the lipid A (**Figure 7**) concerning the substitution with 12:0(2-OH) and identified increased amounts of Ara4N attached to the 4′-phosphate depending on culture conditions.[156,157] However, since Ara4N was present in both, polymyxin susceptible and resistant bacteria, a clear correlation could not be established. The chemical structure of the lipid A first proposed by Bhat *et al.*[157] was established by Kulshin.[158] The main lipid A component of *P. aeruginosa* lipid A is pentaacylated and a minor amount is hexaacylated and contains a nonstoichiometric substitution with Ara4N at the 4′-position. However, upon growth under limiting Mg^{2+} concentrations, Ernst *et al.* found an increased amount of hexaacylated lipid A and the additional presence of lipid A species with Ara4N attached also to the 1-phosphate,[159] as was observed previously for *S. enterica* sv. Typhimurium. More importantly, a novel lipid A was identified as the predominant species which was hexaacylated, but contained a 16:0 secondary fatty acid attached to

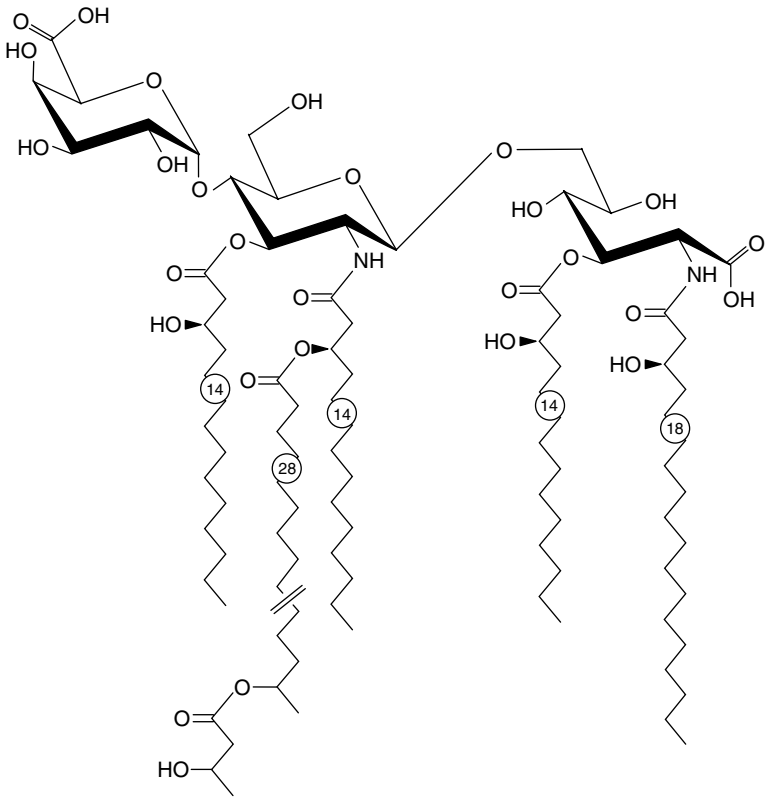

Figure 6 Lipid A from *R. etli*.

the 3′-position instead of the 10:0(3-OH) substitution in position 3. It was suggested that this structural adaptation which occurred in clinical isolates from a cystic fibrosis lung contributed to the increased resistance against CAMP and was the result of PhoP/PhoQ activation.[159] An additional hexaacylated lipid A species was identified in non-mucoid *P. aeruginosa* from blood and mucoid isolates from bronchiectasis which contained a 12:0 secondary fatty acid at position 2 of the lipid A backbone. However, as recently shown, the regulation of cationic peptide resistance in *P. aeruginosa* is very complex and important differences in two-component systems between *Pseudomonas* and *Salmonella* exist leaving space for alternative explanations.[160]

The structural analysis of LPS from *Francisella tularensis* live vaccine strain (ATCC 29684)[161] and *F. novicida* U112[162] indicated that it may lack negative charges completely, apart from a single carboxyl group of Kdo, which connects a short core-OS and the O-PS to the lipid A. Surprisingly, the lipid A was completely devoid of phosphates apart from a very minor amount which contained only a monophosphate at the anomeric position of the reducing GlcN I (**Figure 8**).[161] Other unusual features of this LPS are the lack of a negatively charged substituent at position 4 of Kdo, which has otherwise always been found in LPS, and the presence of free lipid A. The former observation warrants further investigation since chemically labile substituents may have been cleaved off during the preparation of the LPS.[161] However, a recent detailed investigation of lipid A from *F. tularensis* strain 1547–57[163] revealed that in addition to unphosphorylated lipid A a considerable amount of 1-phosphorylated lipid A is present in both strains, and may be further modified by a GalN substitution in strain 1547–57 and *F. philomiragia* ATCC 25015. The conflicting results concerning the phosphate substitution of lipid A in *Francisella* have been suspected to be due to differences in growth conditions, LPS extraction, and lipid A preparation apart from strain-dependent variations.[163] The ValA protein of *Francisella* has been identified as an MsbA ortholog, which transports lipid A with an attached core-OS across the cytoplasmic membrane in an adenosine triphosphate (ATP)-dependent fashion (see below),[164–167] and has been shown to be functional in *E. coli*;[168] thus, the identification of lipid A unsubstituted with Kdo in the outer membrane[161] is surprising considering the normal substrate specificity of MsbA and the biosynthetic pathway in other bacteria. However, mutations in MsbA identified in *E. coli* which were viable only with lipid A without the attachment

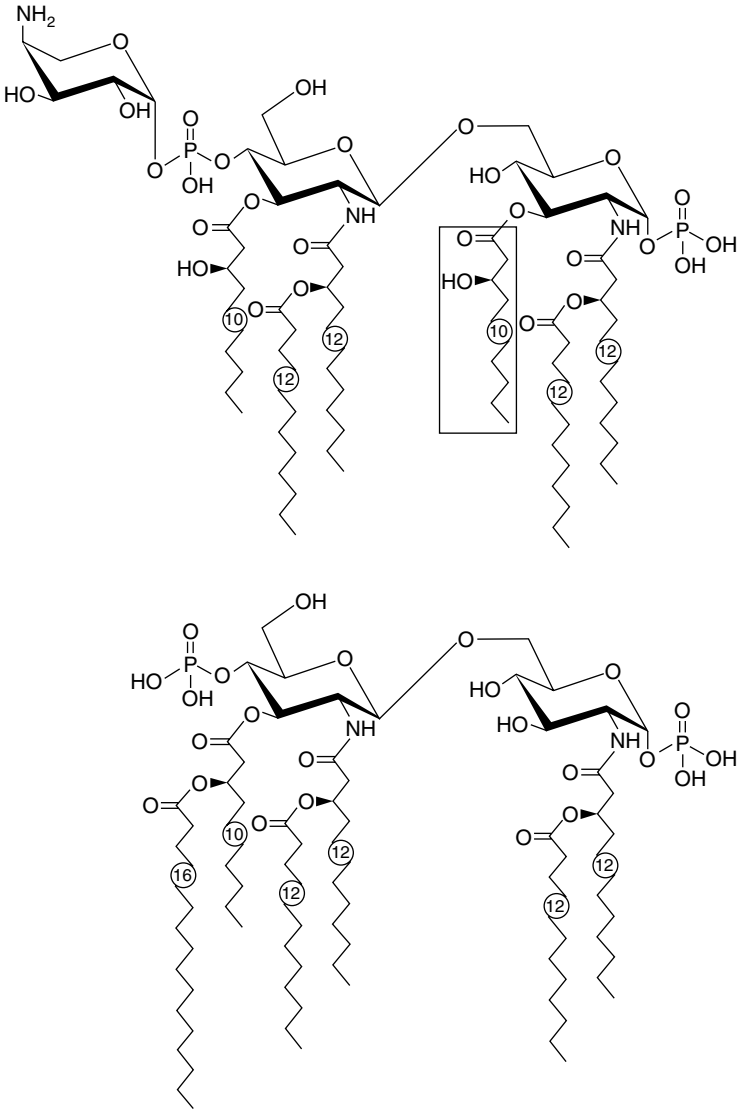

Figure 7 Lipid A species from *P. aeruginosa*.[158,159] The major form is a pentaacylated lipid A shown on the top, in which the 12:0(3-OH) in position 3 of GlcN I is absent (boxed). A *P. aeruginosa* isolated from the airway of a cystic fibrosis patient contained a second hexaacylated lipid A shown on the right.[159] Under low Mg^{2+} conditions lipid A with one and two Ara4N in positions 1 and 4′ or in both positions were found in this study. An additional pentaacylated lipid A species was identified in nonmucoid *P. aeruginosa* from blood and mucoid isolates from bronchiectasis which lacked the 12:0 secondary fatty acid at position 2′ of the lipid A backbone (bottom structure).

of core sugars showed that this is possible in principle.[169] The dephosphorylation of lipid A in *Francisella* is achieved enzymatically by dedicated phosphatases which dephosphorylate the 1- and 4′-position.[170,171] Both enzymes are located in the inner membrane and are suspected to act at the periplasmic face of the membrane after translocation of LPS by ValA. In accordance with the structural requirements for biologically active LPS (endotoxins),[172] *F. tularensis* LPS is biologically inactive and has been shown not to be recognized by the innate immune system.[173]

Different types of lipid A are found in other nonenterobacterial LPS. Structural variability results from differences in the hydrophilic headgroup and in the hydrophobic acylation pattern.[38,80,110,174] GlcN residues constituting the hydrophilic carbohydrate backbone of *E. coli* lipid A may be replaced by 2,3-diamino-2,3-dideoxy-glucopyranose (GlcN3N). The disaccharide has been found to be functionally replaced by a GlcN3N monosaccharide in, for example, *L. interrogans*,[121] *Rhodopseudomonas palustris*, *R. viridis*, *P. diminuta*, and *Phenylobacterium immobilis*[110,174]