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Perspective/Review

Structure of bacterial lipopolysaccharides

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Dedicated to the memory of Dr Alexandre Haeffner

Abstract

Bacterial lipopolysaccharides are the major components of the outer surface of Gram-negative bacteria They are often of interest in medicine for their immunomodulatory properties. In small amounts they can be beneficial, but in larger amounts they may cause endotoxic shock. Although they share a common architecture, their structural details exert a strong influence on their activity. These molecules comprise: a lipid moiety, called lipid A, which is considered to be the endotoxic component, a glycosidic part consisting of a core of approximately 10 monosaccharides and, in "smooth-type" lipopolysaccharides, a third region, named O-chain, consisting of repetitive subunits of one to eight monosaccharides responsible for much of the immunospecificity of the bacterial cell.

Keywords: Lipopolysaccharide; Endotoxin; Structure; Lipid A; Core; O-chains

Contents

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1. Introduction

1.1. History of bacterial lipopolysaccharides

The history of lipopolysaccharides, recently documented by Rietschel and Westphal,[1] began in the eighteenth century with the search for the fever- and disease-producing substance that was associated with unhygienic conditions. This was variously referred to as the pyrogenic material, putrid poison, or toxin. By 1872, the growing awareness of a possible role for living organisms in human diseases allowed Klebs, a German bacteriologist, to attribute a majority of military war deaths to a pyrogenic substance from microorganisms which he called ''Microsporon septicum''. Two years later, a Danish pathologist named Panum reported a non-volatile, heat-resistant, water-soluble, pyrogenic toxin obtained from putrid matter. Later, with the pure-culture techniques developed by Koch,[2] it was possible to show that different diseases were caused by specific bacteria. From the same laboratory in 1892, Pfeiffer reported that the agent of cholera, *Vibrio cholerae*, produced a pyrogenic, non-secreted toxin that was heat-stabile, in addition to a secreted, heat-labile toxin. He called it endotoxin, a term still used today for the lipopolysaccharides that were later found to constitute them. Nevertheless, we know at present that endotoxins are not all toxic, just as bacteria are not all pathogenic. The endotoxins were soon shown to characterize the major group of Gram-negative bacteria, i.e., those having a second, outer membrane.

The development of techniques for extracting and preparing endotoxins sufficiently pure for structural studies was slow. In the 1930s and 1940s, fairly pure preparations were reported to consist of a polysaccharide, a lipid part, and a small amount of protein, and received the name lipopolysaccharide (LPS).[1] The 1950s and 1960s saw the introduction of the extraction methods most commonly used today.[3,4]

The enterobacterial LPSs (especially those of *Escherichia coli* and *Salmonella enterica* serovar typhimurium) were most thoroughly studied. Strains of these species gave colonies with either a rough or a smooth appearance. By 1964, the former were found to produce LPS containing glucosamine, glucose, galactose, the recently characterized L-*glycero*-D-*manno*-heptose and 3-deoxy-oct-2-ulosonic-acid (Kdo), as well as phosphate and the C_{12} and C_{14} lauric, myristic, and hydroxymyristic (C_{14}OH) acids. Strains giving rise to colonies with a smooth aspect also produced LPSs with these components, but with many other sugars as well.

The LPSs resisted structural characterization by their amphipathic nature. Their strong tendency to form aggregates made it difficult to determine their molecular weight. In addition to hydrophobic bonds, there were problems of intermolecular crosslinking of acid groups (phosphates, pyrophosphates, and acid sugars) via divalent cations. Chelation and electrodialysis removed some of the latter as well as monovalent ($Na+$, $K+$) ions.[5,6] Nevertheless, such techniques, with or without the replacement of divalent cations by the more soluble ammonium or triethylammonium salts, were useful in lowering the size of aggregates.[7] Molecular-weight estimation by SDS-polyacrylamide gel electrophoresis, which gave good resolution, suffered from the lack of appropriate molecular-weight standards. In any case, most LPS preparations were (and still are) heterogeneous. Precise molecular masses of native LPS molecules were obtained only after progress was made in the preparation of LPS for mass spectrometry. Nevertheless, the natural heterogeneity of most LPS preparations still complicates the interpretation of their spectra.

The finding that the lipid region could be separated after weak acid hydrolysis helped establish the general architecture comprising two or three regions:[8] a lipid region, a core, and the third region in bacteria yielding smooth colonies. The use of rough mutants having cores of different lengths facilitated the determination of the *Salmonella* core structure.[9,10] In the early 1980s, the structures of both the lipid A and the core were established. The regions going from the bacterial membrane toward the outside are, in order: a lipid (called lipid A) linked to Kdo of the core oligosaccharide, itself linked to the second glycoside if any, consisting of a sequence of repetitive subunits and called O-chain or O-specific antigen (Fig. 1). The endotoxins were later recognized as the matrix of the external leaflet of the bacterial outer membrane. It forms the major component (45%) occupying 75% of the surface of the bacterium. *E. coli* is estimated to have 10^6 molecules per cell.[11] The polysaccharide moiety is directed outwards from the bacterium surface, extending up to 10 nm from the surface (Fig. 1).

The biosyntheses of the lipid A and core are finely orchestrated and the combination put into place followed by the O-chain. The O-chains extending outward from the bacterial cell surface are exposed to the environment and the defence system of a potential host.

1.2. The importance of bacterial lipopolysaccharides

It is not surprising that most LPSs selected for analysis have been those of medical or veterinary interest. In many cases, bacteria that were not considered to be human pathogens were found in infected, immuno-compromised patients. In other cases, bacteria that were pathogenic for other mammals, became pathogens for humans, immuno-compromised or not, and vice versa. At present, considerable attention is being given to the LPSs of Gram-negative, nitrogen-fixing bacteria. These are not pathogens of course, even though one

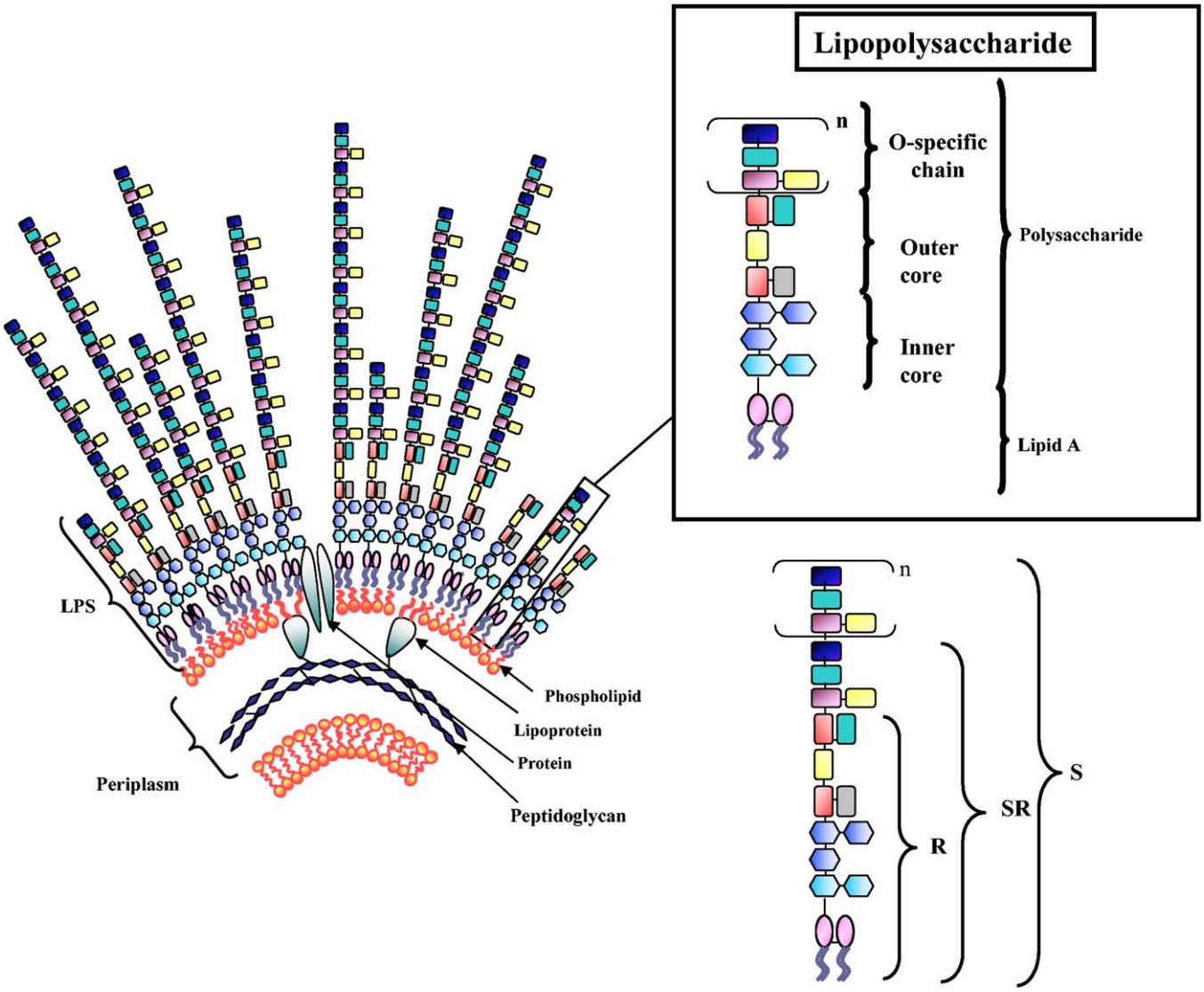


Fig. 1. Schematic representations of the enterobacterial Gram-negative cell envelope (left), a lipopolysaccharide structure (right), R, SR, and S indicate the structures of Rough-type, Semi-Rough type (with only one O-chain subunit) and Smooth-type lipopolysaccharides, respectively.

refers to the interaction between plant and bacterium that results in their symbiosis as an infection.

Although not secreted by the cells, small amounts of the LPS are liberated into the medium under some circumstances such as cell division. Larger amounts are released by bacteria killed by antibiotics, phagocytosis, the complement complex, or treatment with divalent cation chelators. In an infected host, small amounts of LPS can be protective by stimulating the immune system, they have e.g., been used to shrink tumours. Large amounts, however, induce high fever, increase heart rate, and lead to septic shock and death by lung and kidney failure, intravascular coagulation, and systemic inflammatory response.

Most of the biological activities have been associated with the lipid moiety of the molecule. However, the role of the polysaccharide moiety is not negligible. This has been illustrated by the stronger biological activities induced by Re-type LPS consisting of lipid A carrying only two Kdo residues compared to those of isolated or synthetic lipid A.[12,13] The conformation of at least part of the lipid A is modified by these sugars and their charges.[14] A highly purified lipid A was unable to induce secretion of interleukin-1 by human monocytes,[15] and a charged, attached Kdo unit was shown to be necessary for this activity.[16,17] The importance of the polysaccharide moiety also lies in its antigenic properties, its effect on the solubility of the LPS molecule, and its charge. Other activities which require an oligosaccharide include mitogenicity (the stimulation of B cell division) and the activation of human macrophage cell lines.[17,18]

2. Chemical structure of lipid A

In the early stages of endotoxin research, the term "lipid A" referred to a component later recognized as the lipid moiety of the LPS molecule. Another component "lipid B" was later identified as phosphatidylethanolamine,[1] a membrane component often contaminating LPS preparations. The terms "isolated" and "free" lipid A refer to the lipid moiety separated from LPS after mild hydrolysis.[1] Detergent-catalysed hydrolysis helps solubilise the LPS preparations (see Section 5.4).[19]

The first lipid A structures to be established were those of Enterobacteria. This part of the molecule was chemically less heterogeneous than the polysaccharide region. With the determination of some non-Enterobacterial lipid A structures, a common general architecture was established, namely a bisphosphorylated β-(1→6)-linked glucosamine disaccharide substituted with fatty acids ester-linked at positions 3 and 3′ and amide-linked at positions 2 and 2′.[20] Position 4 of $GlcN^{I}$ and position 6 of $GlcN^{II}$ are free, the latter being the attachment site of the glycosidic region. Reviews dedicated to lipid A by Zähringer and co-workers,[20] and to LPSs by Alexander and Rietschel[21] have reported extensive data on the structure of lipid A. The review of Raetz and Whitfield presents the processes of LPS biosynthesis.[11]

2.1. Lipid A acylation

Escherichia lipid A is most frequently described as a hexaacylated molecular species although penta-, and tetra-acylated molecules are also present in varying amounts. The presence of a C_{16} fatty acid linked in secondary linkage (see Section 5.6) forming a heptaacyl lipid A was first described as a characteristic of *Salmonella* lipids A.[22] Comparison of a large panel of *E. coli* and *Salmonella* lipid A structures showed that the heptaacyl molecular species was not especially typical of *Salmonellae* and that the use of the expression "Salmonella-type lipid A structure" should be avoided.[23] It has been shown that the presence of a C_{16} fatty acid in *Salmonella* LPS is regulated by the PhoP–PhoQ system.[24] This is also the case with *Yersinia enterocolitica* and *Bordetella bronchiseptica* lipid A.[25] Such variation is also observed in response to specific aspects of the growth medium (pH, Mg^{2+} concentration, presence of cationic peptides). These factors can also result in modifications in the degree of hydroxylation of the C_{14} fatty acid by Lpx O and of substitution of the phosphate groups.[26]

Heterogeneity in the degree of fatty acid substitution often results in a mixture of three or four molecular species in a single preparation. This heterogeneity may be due to underacylation or to post-synthesis degradation. Underacylation can also be ascribable to mutations leading to a defect in fatty-acid-transferases. The structures described in this review refer to the main molecular species.

The most common fatty acids in lipid A have 10–16 carbon atoms although longer chains exist, i.e., C_{18} fatty acids in *Helicobacter pylori* lipid A, and C_{18} and C_{21} in *Chlamydia trachomatis*.[27–30] Hydroxylated fatty acids are usually found in direct acylation of the sugar except in a few cases (see below) where they appear in secondary acylation. Some *Bordetella* strains have unhydroxylated fatty acids at the C-3 position.[31] In *Chlamydia* lipid A, hydroxylated fatty acids occur only as amide-linked substituents while positions C-3 and C-3′ are both substituted by C_{14} fatty acids (Fig. 2).[32,33] Another kind of fatty acid, which has been described for a few species, is the 3-oxo fatty acid, e.g., the 3-oxotetradecanoic acid found in *Rhodobacter capsulatus*, and the 27-oxooctacosanoic and heptacosane-1,27-dioic acids found in *Legionella pneumophila* (Table 1).[34,35] The number of fatty acid groups present in a molecule has a direct effect on its toxicity, six being the optimum. Fatty acid chain length is also critical for lipid A toxicity since C_{12}, C_{12}OH, C_{14}, and C_{14}OH fatty acids are those found in the most toxic lipids A.[36] Unsaturated fatty acids are rarely present in lipid A, but examples were found in *Rhodopseudomonas sphaeroides* and other species of this genus as well as in *Rhodobacter capsulatus* (Table 1).[37] Another kind of unusual fatty acid, with a methyl branch at C_{n-1} is found in some *Legionella* and *Porphyromonas* strains.[38]

Most hydroxylated fatty acids have the hydroxy group at the C-3 position. However some lipids contain 2-OH-C_{14} fatty acids. Two examples are found in the lipid A of *S. typhimurium* at C-3′ and of *Bordetella hinzii* at C-2′ in secondary acylation, as well as in *Pseudomonas*, *Shigella*, and *Enterobacter* lipids A.[39,40] This peculiar acylation is related to growth temperature variation in most cases,[41] but not in *B. hinzii* (Table 1). In other *Bordetella* species, the same position is substituted by a non-hydroxylated C_{14} fatty acid.

Bordetella lipid A structures have the classical bisphosphorylated β-(1→6)-linked glucosamine disaccharide backbone with two amide-linked C_{14}OH fatty acid substituents. The nature and distribution of ester-linked fatty acids have until now been proved to be species- or strain-specific.[42] An unusual feature compared to most of the structures described, is the absence of symmetry at C-3 and C-3′ positions.[42] The lipids A of some *B. bronchiseptica* strains also display a lack of specificity at C-3, with either C_{12} or C_{12}OH fatty acids. This may be ascribed to a "relaxed" specificity of the UDP-*N*-acetylglucosamine transferase.[43] *B. parapertussis* has a non-hydroxylated fatty acid at this position.[42] This feature has been described in *Chlamydia* lipid A as a strict characteristic.[32]

Yersinia lipid A structures have been neglected until recently, probably because the main structure found in

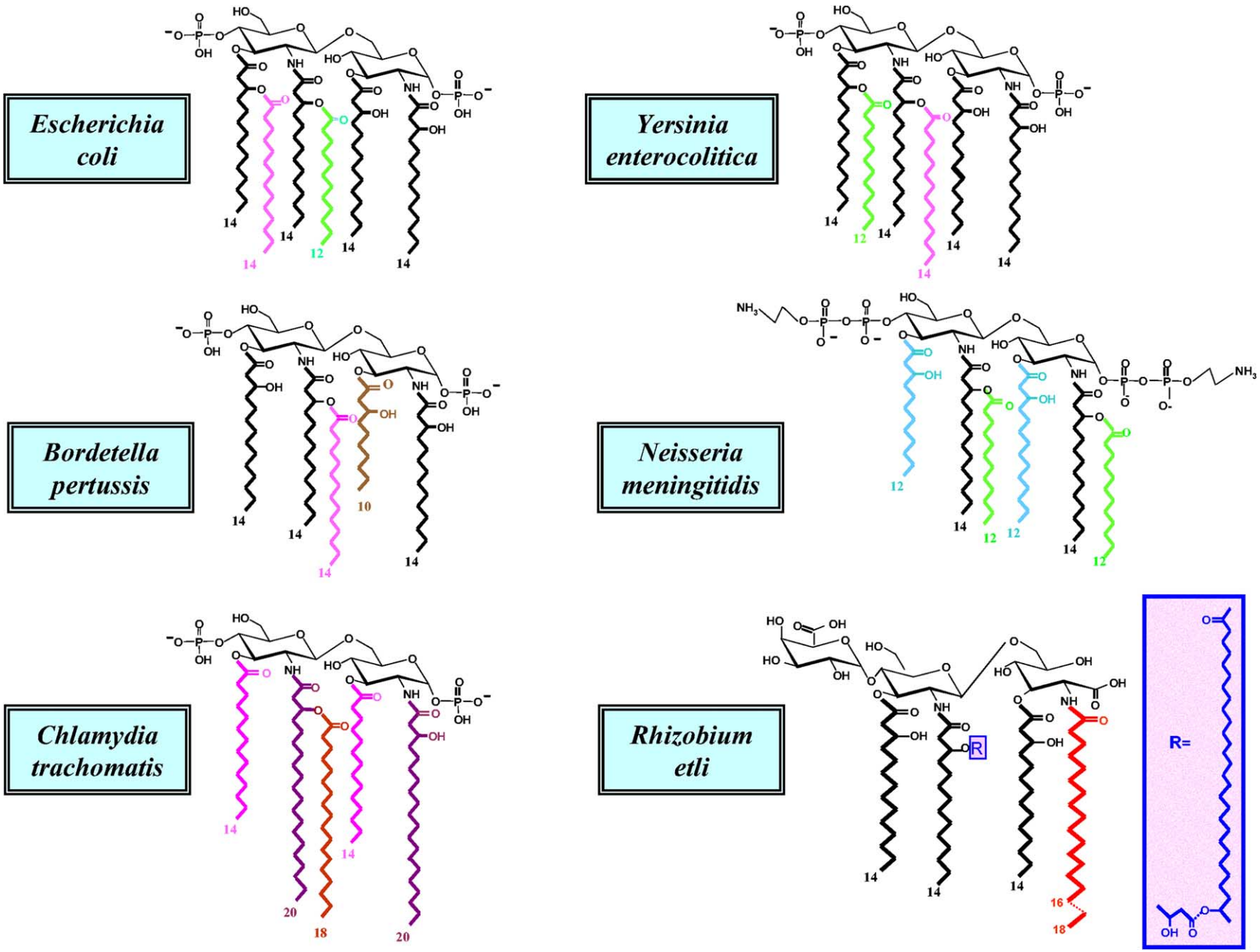
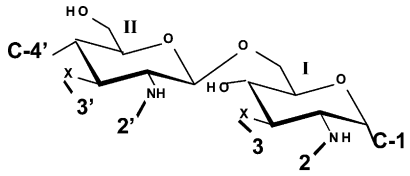


Fig. 2. A selection of lipid A structures. *Escherichia* and *Yersinia* are *Enterobacteria*.

Table 1
Nature and linkage type of acyl substituants and subunits on the lipid A backbone



	GlcN^{II}			GlcN^{I}			Ref.
	C-4′	3′(ester)	2′(amide)	3 (ester)	2 (amide)	C-1	
Bordetella hinzii	P	$C_{14}OH$	$C_{14}O$ C_{14}(2-OH)	$C_{12}OH$	$C_{14}OH$	P	40
Burkholderia caryophylli	P	$C_{14}OH$	$C_{16}OC_{14}$	$C_{14}OH$	$C_{16}OH$	P-L-Ara4N	61
Rhodobacter capsulatus	P-EtN	$C_{10}OC_{12:1}$	C_{14}(3-oxo)	$C_{10}OH$	C_{14}(3-oxo)	P-PetN	34
Yersinia pseudotuberculosis	P-L-Ara4N	$C_{14}OH$	$C_{14}OC_{16}$	$C_{14}OC_{16}$	$C_{14}OH$	D-Ara*f*	46
	DAG ^{II}			DAG ^{I}			
	C-4′	3′(amide)	2′(amide)	3 (amide)	2 (amide)	C-1	
Aquifex pyrophilus	D-GalA	$C_{14}OC_{18}$	$C_{16}OH$	$C_{14}OH$	$C_{16}OH$	D-GalA	143

Abbreviations: Ara4N, 4-amino-4-deoxy-L-arabinopyranose; D-Ara*f*, D-arabinofuranose; DAG, 2,3-diamino-2,3-dideoxy-D-glucopyranose; D-GalA, D-galacturonic acid; P, phosphate; PEtN, phosphorylethanolamine.

Y. enterocolitica has the same molecular mass as found in *E. coli* lipid A.[44] The idea of a common lipid A structure for these two Enterobacteria was proposed but recently corrected.[45] As in *Bordetella* species and strains, lipid A structures of different *Yersinia* species vary, the main difference being for C-2′ fatty acid secondary substitution.[45] The fatty acid is C_{12} for some *Y. ruckeri* strains, C_{12} or C_{14} for some *Y. enterocolitica* strains, C_{16} in one strain of *Y. pseudotuberculosis*,[46] and $C_{16:1}$ for *Y. pestis*. In the latter case, the presence of this fatty acid was found to be dependent on growth temperature, whereas *Y. enterocolitica* required much lower temperatures to add the $C_{16:1}$ substituent.[45,47]

The first *Helicobacter pylori* lipid A described had an unusual structure, hypoacylated with long-chain fatty acids and no phosphate group at C-4′.[27] Later, it was shown that some strains have the phosphate group and a higher degree of acylation.[28] The lipid A of one strain of *Helicobacter mustelae* has the more classical bisphosphorylated backbone, in which the amide-linked fatty acids were variably $C_{14}OH$ or $C_{16}OH$.[48]

2.2. Unusual glycosidic backbones in lipid A structures

The GlcN backbone of some lipids A is replaced by a 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N)-containing mono- or disaccharide. Mixed disaccharide backbones (GlcN3N–GlcN) are found in *Campylobacter*, *Rhodopseudomonas*, and *Thiobacillus*.[49–51] A diaminoglucose disaccharide backbone was reported in *Ochrobactrum anthropi*,[52] and a monosaccharide backbone in lipids A of *Rhodopseudomonas viridis* and *R. palustris*.[37] The lipid A of *Rhizobium trifolii* is distinguished by the presence of D-glucosaminuronic acid (GlcNA).[53] Another interesting structure discovered in the family of Rhizobiaceae, is a diacylated glucosamine backbone with a galacturonic acid residue replacing the phosphate at C-4′ in *R. etli* and *R. leguminosarum*. In some molecular species, the glucosamine^{I} residue is replaced by 2-amino-2-deoxy-gluconate (Fig. 2).[54] All lipid A species of *R. etli* contain a C_{28} fatty acid carrying a β-hydroxybutyrate substituent at C-27.[55]

Phosphate groups and an occasional pyrophosphate are usually present at positions 1 and 4′. However, exceptions exist with no or only one phosphate group or with a negatively-charged sugar.[56] There are a few examples that have neutral sugars.[57] In some cases, the negative charges of the phosphate groups are neutralized by substitution with phosphorylethanolamine, glucosamine or 4-amino-4-deoxy-L-arabinose.[58] Phosphorylethanolamine is a frequent substituent whereas neutral substituents like D-arabinofuranose, L-*glycero*-D-*manno*-heptose, and D-mannose are relatively rare.[20] Accumulation of anionic groups in the lipid and proximal core regions connected by divalent cations to adjacent lipid A molecules makes for a strongly stabilized molecular region. Conversely, the presence of positively-charged substituents on the phosphate

groups increases the resistance of the bacteria to cationic antibiotics.[59] The most commonly described substituents are those present in the lipid A structure of *Salmonella minnesota* Re 595, which include phosphorylethanolamine at the glycosidic position and 4-amino-4-deoxy-L-arabinose on the phosphate at C-4′ of $GlcN^{II}$. These two substituents have been found at these positions in strains of *Yersinia*, but in *Neisseria meningitidis* both phosphate groups are substituted with phosphorylethanolamine,[60] and in *Burkholderia caryophylli*,[61] the lipid A structure shows a 4-amino-4-deoxy-L-arabinofuranose 1-phosphate residue exclusively at C-1 of $GlcN^{I}$ (Table 1).

The 3-D structures of lipid A (conical, cylindrical or lamellar) depend on the presence of the substituents described above. It has been postulated that these various shapes are decisive factors for their endotoxic activity.[62]

A selection of other noteworthy lipid A structures is presented in Table 1.

3. LPS core structures

3.1. Enteric cores

Enteric bacterial LPS cores typically consist of 8–12 often branched sugar units (Table 2). The sugar at the reducing end is always α-3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) (2→6)-linked to $GlcN^{II}$ of lipid A. This bond is sensitive to mild-acid hydrolysis. At the C-4 position of Kdo, there may be one or two Kdo groupings. Three L-*glycero*-D-*manno*-heptose residues (L,D-Hep) are also (1→5)-linked to the first Kdo. A heptose residue may be substituted by a phosphate, pyrophosphate, or phosphorylethanolamine group, or by another sugar to make up the inner core (especially in *Salmonella*, *Escherichia*, and *Shigella*). Such substituents are often present in non-stoichiometric amounts.[63]

The outer core in LPSs of *Salmonella*, *Escherichia*, *Shigella*, *Hafnia*, *Citrobacter*, and *Erwinia* generally consists of an oligosaccharide (up to six sugar units) (1→3)-linked to Hep^{II} (counting from the reducing end of the core). It is often branched with Glc, Gal, or their derivatives. Exceptions are found in the outer core of *E. coli* K12 which includes a unit of L,D-Hep, and strains of *Klebsiella pneumoniae* with one or two units of D-*glycero*-D-*manno*-heptose (D,D-Hep) linked to a Kdo unit which serves as the attachment site of an O-chain.[64,65]

In the three best-studied species of the genus *Proteus* (*vulgaris*, *penneri*, and *mirabilis*), the inner core of almost all strains have their Kdo^{I} residue substituted at the C-8 position by 4-amino-4-deoxy-β-L-arabinose. Otherwise, this part of the core resembles that of other enterics. The outer cores, however, vary considerably. A partial list of their unusual constituents includes a Kdo linked to Hep^{III}, a GalA amide-linked to an aliphatic amine or to the α-amino group of L-lysine, 2-glycylamino-2-deoxy-D-glucose, the open-chain form of GalNAc glycosidically linked to GalN in the form of a cyclic acetal.[66–68]

3.2. Non-enteric cores

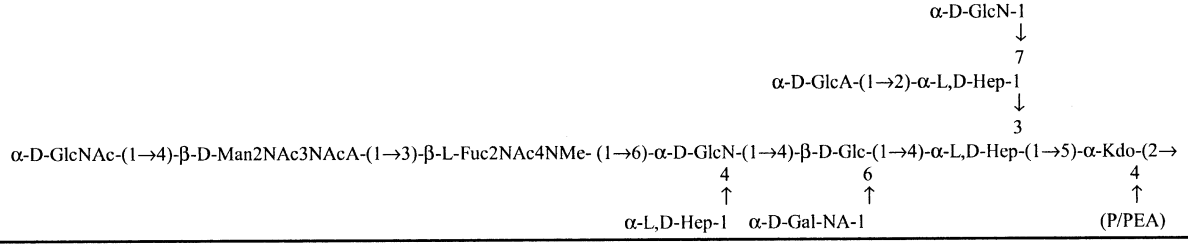
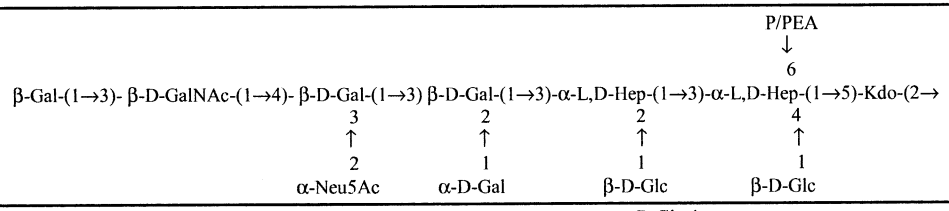
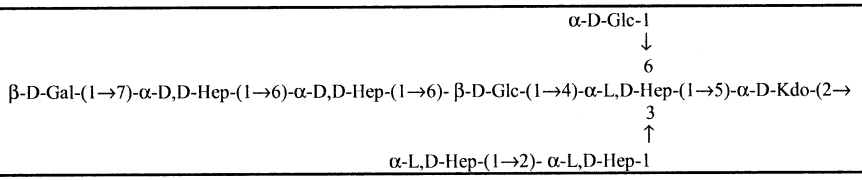
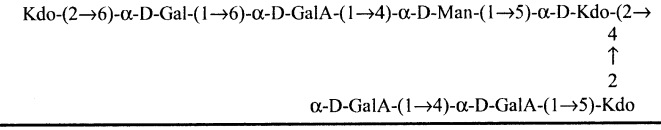
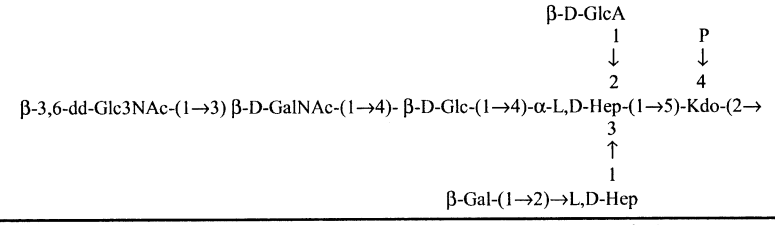
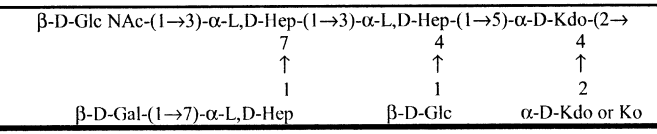
A core that has been reported to be entirely lacking heptoses components is that of *Francisella tularensis*, the agent of tularemia. The one Kdo unit present is linked to a β-mannose-(1→4)-α-mannose disaccharide substituted at O-3 and O-2 by α-Glc and α-GalN-(1→2)-β-Glc, respectively.[69] The entire hexasaccharide has thus only one negative and one positive charge. Since the lipid A is reported to be without phosphate, the outer membrane of this *Francisella* species might be expected to be relatively unstable.[70] Another core which does not contain a heptose is that of *Legionella pneumophila* which, like *Francisella* has its Kdo residue linked to a mannose residue.[71] The rest of the core is noteworthy for the presence of its O-acetyl groups on all of the five remaining sugars as well as three N-acetyl groups. The rough strain of *Ochrobactrum anthropi* LMG 3301 is another example of a core which does not include heptose.[52] Its genus has the relatively rare lipid A backbone in which GlcN is replaced by GlcN3N.

Other genera that produce LPS cores devoid of heptose include the pathogens *Acinetobacter* (of which one species has a core containing 3-deoxy-D-*lyxo*-hept-2-ulosaric acid), *Chlamydia*,[30] and *Moraxella*,[72] the plant symbionts *Rhizobium* and *Bradyrhizobium*,[73] as well as *Bacteroides fragilis*, *Rhodobacter*, etc.[63] *Rhizobium etli.* has a Kdo at the non-reducing end of the core (like *Klebsiella*, Table 2).[73]

Three bacterial species synthesize and incorporate the Kdo analogue D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko): *Burkholderia cepacia*, *Acinetobacter haemolyticus*, and *Yersinia pestis*.[63,74] The Ko can be incorporated instead of Kdo or in addition to it.

In the early compositional analyses of enteric LPSs, Kdo and Hep, along with hydroxytetradecanoic acid were considered to be identifying characteristics of LPS. They are present in LPSs of wild-type enteric bacteria, but the production of mutants with shorter cores showed that these bacteria could survive under laboratory conditions without the heptose.[9] Later, several non-enterics were found to be devoid of heptose, and a few had replaced Kdo by Ko. More recently, a *Neisseria meningitidis* (a non-enteric) mutant lacking a core was made and not only survived but could grow slowly in the laboratory.[75] The presence of a particular capsule of α-(2→8)-linked *N*-acetyl-neuraminic acid (Neu5Ac) is thought to protect the bacterial outer membrane. Some non-enteric Gram-negatives (*Vibrio cholerae*,

Table 2
A sampling of lipopolysaccharide core structures

		Ref.
Bordetella pertussis 1414		80
Campylobacter jejuni NCTC11168		144
Mannheimia haemolytica A1		84
Rhizobium etli CE3		73
Vibrio parahaemolyticus O12		145
Yersinia pestis		74

Abbreviations : dd, dideoxy ; Fuc2NAc4NMe, 2-acetamido-4-*N*-methyl-2,4,6-trideoxygalactose; GalNA, galactosaminuronic acid; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; L,D-Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid; Man2NAc3NAcA, 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid ; Neu5Ac, *N*-acetylneuraminic acid.

Bordetella pertussis, *Haemophilus*, etc.) were thought to be devoid of Kdo residue because it was not detected by the commonly used assay method. This turned out to be wrong: their Kdos were substituted at O-4 by a phosphate group which was eliminated during the hydrolysis step with formation of a double bond.[76,77] This product reacted poorly with the colour reagent.

More diversified sugars are found in the outer cores of non-enteric bacteria, but the inner cores of most analysed LPSs do have Kdo and heptose.

The great majority of sugars encountered in LPSs are hexoses in the pyranose form. Some exceptions are with 3-deoxy-α-D-*manno*-oct-2-ulofuranosonic acid in the *Aeromonas salmonicida* core, rhamnose in some *Pseudomonas aeruginosa* strains, and fructofuranose and sedoheptulofuranose in *Vibrio cholerae*.[63]

Considering *Bordetella* cores, in *B. pertussis*, the agent of whooping cough, only one Kdo is present. In about half of the LPS molecules, it is phosphorylated at C-4. About 90% of the core is a dodecasaccharide but the rest is a nonasaccharide (these correspond to the A and B bands, respectively, in SDS-polyacrylamide gels).[78,79] The two heptoses are not phosphorylated but the branching Hep^{II} is doubly substituted with a hexuronic acid and an amino sugar. Distal to Hep^{II} are a Glc residue, substituted with a hexosaminuronic acid, and a GlcN residue which itself is substituted by a terminal heptose and by the terminal trisaccharide motif comprised of α-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAc3NAcA-(1→3)-β-L-Fuc*p*NAc4NMe (Table 2).[80] The latter is encoded by a cluster of genes separated from the cluster encoding the rest of the core and represents an important antigen in pertussis.[79,81]

Bacterial strains *B. bronchiseptica* and *B. parapertussis*, which are sometimes considered to be subspecies of *Bordetella* genus, produce smooth-type LPSs.[82] The LPS core of the former is similar to that of *B. pertussis* except for the presence of non-stoichiometric amounts of the terminal outer-core heptose in some strains. The *parapertussis* core differs in that cores without O-chains lack five terminal sugars whereas cores bearing O-chains lack the terminal GlcNAc.[83]

The core oligosaccharide component of *Mannheimia* (*Pasteurella*) *haemolytica* serotype A1 LPS contains L,D-heptose and D–D heptose: its structure is shown in Table 2. The genus *Mannheimia* is related to *Haemophilus*. As in other LPS cores containing the two heptose forms, the inner core has L,D-Hep residues (three in this case) and the outer core has the D,D-Hep residues. The two forms of Hep are separated by one glucose unit, and the rest of the core consists of another Glc residue linked to L,D-Hep^{I} and a galactose unit linked to D,D-Hep^{II}. No charged groups were reported except for the one in the Kdo unit.[84]

In *Pseudomonas* cores, one often finds an alanyl group substituting a GalN residue, and a carbamoyl group on $heptose^{II}$.[63] *P. aeruginosa* PAO1 serotype O5 and its two rough-type mutants share these characteristics, but have in addition three phosphomonoester groups on their $heptose^{I}$. It was suggested that these groups play a role in interactions with antibiotics.[85,86]

Since it was not easy to determine the precise location of phosphate, pyrophosphate, and phosphorylethanolamine groups, this information was often not given in early reports although it is important for determining the distribution of charges on the core and for the explanation of underestimates of Kdo.[76,77] In the latter case, the Kdo-lipid A bond is strengthened by the presence of the phosphate group. Phosphate groups and charged sugar residues in the inner core and lipid A are assumed to contribute to the stability of the outer membrane via crosslinking cations. However, there are wide variations in the number of negatively-charged components: one to three units of Kdo, the presence or absence of phosphate groups on lipid A, on Kdo and/or on heptoses, and of an occasional hexuronic acid. In view of the great variability in density of these charges in the lipid A-inner core region of different bacteria, it may be advisable to take into consideration the specific environmental conditions encountered by the bacteria, as well as the nature of their capsule, (if any) to explain the diversities.

In general, a bacterial species is likely to have many more O-chain types than core types. A study of the distribution of the five *E. coli* core types among 72 isolates representative of the four phylogenetic groups showed no strict association of any core type with one group.[87]

3.3. Heterogeneity of cores

The degree of heterogeneity in the core of a bacterial strain depends partly on the number of non-stoichiometric components present. In *Bordetella pertussis* 1414 core, there are two such components: phosphate/phosphorylethanolamine groups on the Kdo residue and the presence or absence of the terminal trisaccharide. Although frequently attributed to incomplete biosynthesis, non-stoichiometry could also be due to partial degradation after complete biosynthesis. Variation in LPS core structures in response to specific aspects of growth medium, already mentioned in the section on lipid A, have neither been investigated, e.g., the phosphate, pyrophosphate, or phosphorylethanolamine groups on Kdo and heptoses, terminal sugars, amino acid, carbamoyl, and other groups occurring non-stoichiometrically.

Another kind of core heterogeneity, called "phase variation", has been studied particularly in the O-chainless genera of *Haemophilus*, *Neisseria*, *Campylobacter*, and other pathogens that invade mucosal tissues.[88–91] The terminology "phase variation" applies

also to heterogeneities in bacterial cell-surface components other than LPS cores. The basis for this kind of variation in species of the first two genera mentioned is thought to be polymerase slippage during replication of short nucleotide repeats in genes encoding biosynthetic enzymes. This shifts the reading frame on the DNA and results in a switch-like change in the synthesis and addition of certain groups of sugars to a core. The best known examples of these are the lacto-*N*-neotetraose [β-Gal(1→4)β-GlcNAc(1→3)β-Gal(1→4)β-Glc] and digalactoside of *Neisseria*. The variably expressed terminal neotetraose unit of many pathogenic *Neisseria* LPS cores is identical to the terminal oligosaccharide of human paragloboside which, when attached to a ceramide, is the human I and i blood group antigens. In *N. meningitidis*, the β-Gal is sometimes sialylated and the degree of sialylation of the core terminal seems to be varied in order to enhance the invasiveness of the bacterium and improve survival in the host. In another strain, L1, the α-D-Gal terminal of an antigenic trisaccharide can be sialylated at O-6.[92] *Haemophilus influenzae* strain Road, grown on sialic acid-containing medium, produced sialylated lacto-*N*-neotetraose linked to the inner-core heptose^{I}. The complete oligosaccharide was either present or absent, which was not the case with *Neisseria*.[92] Conversely, strain RM7004 of *H. influenzae* makes a great variety of glycoforms with different substituents linked to heptoses^{I,II}, some of which include the tetraose as terminal unit on both of these heptoses.[91] The consequences of such variations for the invasiveness of these strains has not been determined.

An estimation that replication slippage involves 1 in 10^2 to 10^3 cells is believed to be sufficiently frequent to allow the invader to survive and flourish in a host. Another example of phase variation involving adaptation of LPS core structure in an infection context is that of the cores of *Campylobacter jejuni* (Table 2). These bacteria invade and cause tissue damage in gastrointestinal infections by way of ganglioside mimicry. The structural variations may have any of several types of genetic origin. Replication slippage is one of them.[93] The result of such modifications in core structure is either a masking of an antigenic group (epitope) on the core or a mimicking of a host structure. In both cases, the effect is a dulling of the potential reaction of the host.[94]

4. The O-specific chains

4.1. Functions and generalities

O-Specific chains are present only in smooth-type Gram-negative bacteria. They consist of repetitive subunits which make polysaccharides extending out from the bacteria. In pathogens, these O-chains are in direct contact with the host during infection. Since they are antigenic, they form the basis for serotype classification among the various bacterial families. Interestingly, these chains help the bacterium to escape the lytic action of the complement complex by a "shielding" process.[95] They also protect the bacteria from the effect of numerous antibiotics as shown by the relative sensitivity of rough-type strains as compared to smooth-type bacteria.[96,97] The O-chains of *Actinobacillus pleuropneumoniae* and of some other LPSs have been shown to be responsible for adhesion to mammalian tissues thus favouring infection. In lung tissues, the LPS of *A. pleuropneumoniae* is the major adhesin.[98] Some O-chains can even adhere to a mineral surface as in the case of *Shewanella algae* LPS (this LPS was the first bacterial polysaccharide found to contain a residue of malic acid).[99]

The O-chains determine the specificity of each bacterial serotype, a kind of fingerprint for bacteria. A combination of monosaccharide diversity, the numerous possibilities of glycosidic linkage, substitution and configuration of sugars, and the genetic capacities of the diverse organisms, have all contributed to the uniqueness of the great majority of O-chain structures.

The biological interest of O-chain polysaccharide structures as well as their diversity are subjects too extensive to cover in this review. We present here a short selection of some recently published and unusual structures. Other examples of O-chain structures recently reported are listed in Table 3. For still more O-chain structures, see the reviews of Kenne and Lindberg,[100] Knirel and Kotchetkov,[101] Jansson,[102] and Raetz and Whitfield.[11] Diverse internet sources can also provide much information.

O-chain complexity and adaptation are encountered in the *Helicobacter* polysaccharides, which mimic Lewis blood-group antigens as described in a recent review by Monteiro.[103]

O-Chain structures, like any polysaccharide structures, can be linear or branched and substituted by many different aglycones (Table 3). The most common substituents are O- and N-acetyl, phosphate, and phosphorylethanolamine groups.[104] Amino acids in amide linkages, acetamidino groups as well as formyl groups, and glyceric acid are often found as non-stoichiometric substituents.[102]

As stated earlier, each subunit comprises one to eight sugar units and there may be up to 50 identical subunits in an O-chain. During biosynthesis, subunits are polymerised into blocks of varying length and then added to the core. The resulting diversity of chain lengths on different LPS molecules in a culture is responsible for the well-known ladder-like pattern of LPS molecules on SDS-electrophoretic gels.[105] The nature of the O-chain may, in some cases, be directly related to the pathogenic effects of the bacteria. In the O-chains of *Proteus*, acidic

groups such as uronic acid, phosphate groups, and lactic acid occur frequently. It has been suggested that this characteristic may contribute to the capacity of the bacteria in the bladder or kidney to form stones in these organs.[106]

Some bacteria display shorter O-chains on average than others. The smooth-type *Bordetella* species are in this category.[42] In some cases, the last sugar of the O-chain (non-reducing end) carries a substituent blocking the addition of subunits as a terminal signal. The latter can be a simple methyl group or a different terminal sugar with other substituents in a homopolymeric O-chain structure. An *O*-methyl substituent present on the terminal perosamine residue of the O-chain of *Vibrio cholerae* serotype O1 Ogawa differentiates it from the O1 Inaba serotype. This is an example of an important role of the non-reducing end groups in serological reactions.[60,107,108]

4.2. O-chain structural diversity

The *Bordetella* O-chain structures, unlike those of other bacterial genera, show less diversity and a true family likeness. The O-chain structures analysed so far are those of *B. bronchiseptica*, *B. parapertussis*, *B. avium, and B. hinzii*,[82,109–111] (Table 3) and all contain one or two of three isomer forms of a diaminohexuronic acid bearing various substituents on the amino groups. *B. bronchiseptica* and *B. parapertussis* O-chains have the same overall structure which is a further confirmation for a common origin. They have been described as subspecies.

Some O-chains are made of repetitive sugar units interlinked with other compounds like hydroxybutyric acid or diamino-3-deoxy-nonulosonic acid. A disaccharide repeating unit in the *Flexibacter maritimus* O-chain structure contains a trideoxyglucose linked via an (*S*)-2-hydroxyglutaric acid residue to a nonulosonic acid substituted by a hydroxybutyramido group.[112] A similar component, (*R*)-malic acid was recently reported in the O-chain of another fish pathogen, *Flavobacterium psychophilum*.[113] Some O-chains are of the teichoic-acid type, as in *Hafnia alvei*, with glycerol phosphate or, as in *Fusobacterium necrophorum*, with a phosphorylated aminopentonic acid.[114,115]

Deoxysugars are frequent components in O-chain structures, but sugars that are more characteristic of the inner core region like heptose are seldom present.[102]

Although a broad diversity seems to be the rule in bacteria, serological cross-reactivity sometimes indicates similarities or identities between O-chain structures, e.g., *Brucella abortus* and *Yersinia enterocolitica* O:9 have an identical homopolymeric O-chain structure composed of 4-amino-4,6-dideoxy-L-mannose (L-perosamine) residues.[116,117] Also, the same structure with different *N*-acyl substituents has been reported for *Vibrio cholerae* Ogawa and Inaba strains.[118,119] Again, L-perosamine found in the trisaccharide repeating unit of the enteric pathogen *E. coli* O157:H7, causing haemorrhagic colitis and uraemic syndrome, is responsible for the serological cross-reaction with the strains cited above. *Salmonella* O:30 and *Citrobacter sedlakii* also have identical O-chain structures.[120–122] On the basis of O-chain identities, a vaccine of live *S. landau* was elaborated and gave good results against *E. coli* O157:H7 in a mouse model.[123]

5. Personal comments on some methods used in LPS analysis

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) complement each other in resolving almost all structural problems at the milligram level. A good description of these techniques in lipid A analysis is given in a review by Zähringer and co-workers.[20]

5.1. Mass spectrometry

Various soft-ionisation mass spectrometric methods developed in the 1980s have been used to analyse LPSs and their fragments: fast-atom bombardment (FAB), laser-desorption, plasma-desorption, electrospray, and matrix-assisted laser desorption/ionisation (MALDI). The amphipathic and heterogeneous character of the LPS forced the development of new ways of preparing samples.[7,124–126]

Plasma desorption mass spectrometry (PDMS) allowed the analysis of intact LPS molecules and gave good proportionality between the abundance of molecular species in the sample and the intensity of the corresponding peaks obtained.[23] Fragmentation patterns in lipid A molecules were found to be comparable to those obtained with FAB.[127] However, since the plasma source (^{252}Cf) was dangerous to handle, the method was abandoned in favour of MALDI. The latter is more efficient in terms of time and sensitivity, but since the efficiency of desorption of molecular species in a mixture depends on the choice of the matrix, the amounts of some of the molecular species may be underestimated. MALDI provides information on the length of O-chains and the exact number of repeating units.[110] Citric acid has been shown to increase the solubility of LPS molecules.[126] In MALDI, it also increased the desorption/ionisation of unmodified LPS when added to the DHB matrix at a concentration of 0.1 M.

Lipid A spectra are also obtained by fragmentation of native LPS in the mass spectrometer. In this case, its molecular mass can be expected to be more reliably estimated than after hydrolysis.[79]

Table 3
A sampling of lipopolysaccharide O-chain structures

		Ref.
Bordetella avium ATCC5086	→4)-2-*N*-acetamidino-3-[3-hydroxybutanamido]-2,3-dd-β-D-GlcA-(1→	109
Escherichia coli O157:H7 *Citrobacter sedlakii* *Salmonella* O:30 Serogroup N	→4)-β-D-Glc-(1→3)-α-D-GalNAc-(1→2)-α-D-Rha4NAc-(1→3)-α-L-Fuc-(1→	121 122
Hafnia alvei 10457	→6)-β-D-Gal*f*-(1→3)-β-D-GalNAc-(1→3)-β-D-Gal-(1→ 6 ↑ 2 α-NeuAc	146
Legionella pneumophila Serogroup 1	5-acetimidoyl(*N*-methyl)amino-7-acetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid	147
Shewanella algae BrY	→3)-α-D-BacNAc4NHbu-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→2)-L-malyl-(4→2)-α-L-FucN-(1→	99
Yokenella regensburgei	→3)-α-D-FucNAc-(1→2)-L-α-D-Hep-(1→3)-6-d-α-L-Tal-(1→	141

Abbreviations: BacNAc4NHbu, 2-acetamido-4-[D-3-hydroxybutyramido]-2,4,6-trideoxy-D-glucose; Kdo, 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid; NeuAc, acetamido-neuraminic acid; NonlA, non-2-ulosonic acid; Rha4NAc, 4-acetamido-4,6-dideoxy-D-mannose.

5.2. Nuclear magnetic resonance spectroscopy

NMR spectroscopy is still a first-choice method for the determination of O-chain structures, especially when there are many repeating units of fewer than eight sugars since it is relatively easy to get good spectra with a small amount of polysaccharide. In most cases, the attached core gives only weak signals and can be neglected. However, with some bacteria, like the *Bordetellae*, the mass of the O-chain is limited to 6–7 kDa and the core signals are strong enough to interfere with those of the O-chain sugars. Sometimes, the core can be removed by HF treatment or periodate oxidation if the O-chains are resistant to these chemical treatments.[82,109]

5.3. Extraction and purification

When certain hydrophobic LPSs are extracted by the phenol–water method, some or all of it may be found in the phenol layer.[116,117] Prudence therefore dictates that both phases be checked for LPS content. Contamination of LPS preparations with phospholipids, nucleic acids and lipoproteins remains a critical problem, especially with commercial samples used for structural and biological purposes.[23,128]

Three difficulties must be overcome before determining structure/activity relationships in endotoxin research. The first difficulty is to obtain a pure preparation, the second one is to determine the composition in molecular species of the given heterogeneous LPS preparation, and the last is to obtain a reasonable degree of solubility. When these problems are solved, structure analyses can proceed. As a supplementary reward, biological activities measured in different laboratories would, in principle, be easier to compare.

The most popular commercial LPS preparation used by biologists is that of *S. minnesota* Re 595 because of its supposed structural simplicity. It is the smallest LPS (lipid A and two Kdo residues) found in reasonably viable cells. In fact, different lots from a single or different commercial sources can be very heterogeneous as shown by PDMS analysis.[23] The variability lies in the number of fatty acids as well as the presence of substituents such as phosphorylethanolamine and aminodeoxyarabinose. Commercial preparations are often contaminated with phospholipids, DNA, RNA and/or proteins and, in one case was not even the listed LPS type.[23] Samples should at least be checked by TLC, if mass spectrometry is not easily available. TLC is useful for Re (lipid A and two Kdo residues) to SR-type (lipid A, a complete core, and one O-chain subunit) lipopolysaccharides.[129]

5.4. Hydrolysis

The use of excessively long and strong hydrolytic conditions to split the lipid A–polysaccharide bond results in some dephosphorylation and O-deacylation of lipid A.[130] Reports of lipid A structures without the glycosidic phosphate are suspect. Such modifications strongly diminish the biological activities of lipid A as shown, for example, by the loss of toxicity and pyrogenicity of the *B. pertussis* lipid A following release of the glycosidic phosphate.[15] Mild hydrolysis conditions at pH 4.5 in sodium acetate buffer were efficient in many cases.[131] However, they were usually improved by combining with a detergent. SDS-Promoted hydrolytic conditions in particular allow the use of mild conditions to split the ketosidic bond of Kdo in highly aggregated preparations.[19] The method is also valuable when O-chain polysaccharides have acid-labile constituents or, as in the case of *Citrobacter rodentium* LPS, to prevent cleavage of the phosphate diester on the O-chain.[132]

In case of incomplete hydrolysis and a poor yield of lipid A, the EtOH extraction of SDS should be done at about 10 °C to reduce the possibility of solubilising the lipid in EtOH. To maintain the pH at 4.5, the concentration of the buffer should be sufficient, at least 20 mM. Since the optimum conditions for hydrolysis depend on the structure of the molecule, they may require longer or shorter times of hydrolysis. Cleavage kinetics should be done in this case.

5.5. Liberation of polysaccharide and lipid A by a β-elimination reaction

This procedure based on the periodate cleavage within the Kdo free diol and mild alkali treatment at room temperature is useful to obtain well-preserved lipid A, especially those with pyrophosphate substituents in glycosidic linkage. It is an alternative to acid hydrolysis under problematic conditions.[133]

5.6. Lipid A selective sequential release of ester-linked fatty acids

Fatty acid esters in direct linkage to the lipid A backbone sugar (primary esters) are selectively released in mild alkaline conditions without removal of esters linked to the 3-hydroxyl groups of the primary fatty acids (secondary esters).[127] This can be useful in structure analysis. The released fatty acids can be identified by the change in mass of lipid A and confirmed by GLC-MS. The latter step is important to distinguish, for example, 2-OH from 3-OH fatty acids because they cannot be distinguished by a quick MALDI analysis. It also confirms that the esterified acyl-oxyacyl fatty acids (if any) were released intact and reassures that this method is reliable. Lipid A structures

are thus easily determined in combination with PDMS and/or MALDI mass spectrometry.[40,45,46,48,127] Now that the use of hydrazine is restricted for security reasons, the secondary acyl groups can be liberated by stronger treatment with ammonia or triethylamine, which can be evaporated easily from these small samples before the mass spectrometry step.

5.7. Chromatography

TLC using different proportions of isobutyric acid and (M) NH_4OH is a useful method.[129] Fine-grain silica plates with the above solvent yields information on heterogeneity and purity much more quickly than SDS-gel electrophoresis for R- and SR type LPSs. The possibility of using different detection methods such as ninhydrin, charring, and serology makes it even more valuable.[134] The same conditions applied to column chromatography achieves separation of O-chain-linked LPS molecules from R-type LPS in a single preparation.[135] This separation facilitates the observation of structural differences between the cores and lipids A of the two fractions.[83]

Finally, these TLC conditions can be used with samples of lyophilised cells suspended in solvent. One thus obtains data on the mg dry-weight scale of cells. The sample collected from the dried plate after non-destructive visualisation can be analysed directly by MALDIMS.[126]

6. Perspectives

New kinds of MS analysis allow the soft desorption/ionisation of presumably complete high-molecular weight LPS molecular species. By comparing the major mass with the sum of the masses of the separated glycose and lipid moieties, it is possible to detect any component or structure(s) that might have been lost during the hydrolytic process. The acid-labile Kdo bond, which was first thought to be present only at the reducing end of core structures, has been found elsewhere in the core, as in *Klebsiella pneumoniae*.[65] However, all structures are not equally amenable to mass spectrometry. Improvement of techniques in these cases is still needed.

Among recent developments in technology, different methods applied to LPS structural analysis have already been reported.[20] In our opinion, two methods stand out for their promise in this domain, i.e., ion-mobility-mass spectrometry and high resolution magic angle spinning NMR spectroscopy.

6.1. Ion mobility-mass spectrometry (IMMS)

IMMS is an increasingly used method in which separation is coupled with mass spectrometry for the characterization of biomolecular conformations in the gas phase.[136–139] It has been used for the analysis of peptide mixtures using MALDI, and to study non-covalent interactions.[140] Electrospray ionisation sources have also been used in combination with IMMS in order to develop the method for high throughput analysis of complex peptide mixtures. Ion mobility (IM) separates ions on the basis of their collision cross-section which is dependent on the gas-phase conformation of the ion, thus giving structural information. IMMS gives a new dimension to mass spectrometry. The IM part of the technique is equivalent to instant gas-phase chromatography. It separates molecules with the same mass but different conformations or compositions: phosphorylated and non-phosphorylated molecules and others such as drugs, DNA, peptides or lipids. Preparations containing different composition and conformation are seen along ion groups that have different slopes in the 2D contour plots of ion mobility versus mass. To demonstrate the efficiency of the method, IM-MALDI-TOFMS was used to separate and characterize ions originating from monophosphorylated *E. coli* lipid A mixed with a sulphated peptide of similar mass (A. S. Woods and J. A. Schultz, personal communication). Many applications of the method are expected for LPS research.

6.2. High-resolution magic angle spinning method

A recent method used in NMR analysis gives O-chain polysaccharide spectra in isolated LPS or even directly from the surface of bacterial cells.[141,142] For the moment, this approach is restricted to certain bacteria having long, flexible chains of polysaccharides but it holds promise for the future. Its capacity to provide a way of comparing isolated LPS and polysaccharides and their native counterpart on the cells is already very valuable. It is a potential tool for use in fast fingerprinting of bacterial or other cellular antigens.

Since the elucidation of the biosynthetic pathway of *E. coli* LPS, which marks a milestone in LPS research, similar studies are being pursued on other bacteria. Genetic, enzymologic and structural studies show that the overall pathways are relatively conserved. It is expected that the determination of critical enzymes in the pathways will allow the development of new strategies in vaccine and antibiotic research and provide new targets against inflammation.[11]

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