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Lipopolysaccharides: structure, function and bacterial identification☆,☆☆

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Abstract – Lipopolysaccharides (LPS) are the main components of the outer membrane of Gram-negative bacteria. They are glycolipids containing a lipid moiety called lipid A, more often made of a bis-phosphorylated glucosamine disaccharide, carrying fatty acids in ester and amide linkages. Lipid A is linked to a core oligosaccharide of about 10 sugars, substituted in the wild-type strains, by long-chain oligosaccharide repetitive units, extending outside the bacteria and representing their main antigens. In addition to determine the serotype of the bacterium, LPS are highly potent biological molecules, capable of eliciting at the level of minute amounts, beneficial, as well as deleterious activities.

Keywords: lipopolysaccharide / endotoxins / inflammation / structure-activity / serology

Résumé – Lipopolysaccharides : structure, fonction et identification bactérienne. Les lipopolysaccharides (LPS) sont les composants majeurs de la membrane externe des bactéries à Gram négatif. Ce sont des glycolipides comprenant une région lipidique appelée lipide A, le plus souvent faite d'un disaccharide de glucosamines phosphorylées et portant des acides gras en liaison ester ou amide. Le lipide A est lié à un core oligo-saccharidique d'à peu près dix sucres, substitué, pour les souches sauvages, par une longue chaîne faite d'unités d'oligosaccharides répétitifs qui s'étendent à l'extérieur de la bactérie et qui représentent leur antigène majeur. En plus de déterminer les sérotypes bactériens, les LPS sont des molécules hautement actives capable de déclencher à très faible dose des activités aussi bien bénéfiques que délétères.

Mots clés : lipopolysaccharide / endotoxines / inflammation / structure-activités / sérologie

1 Introduction

Lipopolysaccharides (LPS) are the molecular constituents of the so-called endotoxins. LPS are present in the outer leaflet of the external membrane of Gram-negative bacteria. Some examples of the latter are *Escherichia coli*, *Salmonellae*, other Enterobacteriaceae like *Yersiniae* or *Shigellae*, *Enterobacter*, *Proteus*, and pathogens like *Vibrio cholerae*, *Yersinia pestis*, *Brucella abortus*. A single *E. coli* cell is known to contain 2×10^6 LPS molecules, which corresponds to an amount of about 20 femtograms (Minabe *et al.*, 1994).

LPS are made of three different regions (Fig. 1). The first one, a glycophosholipid moiety called lipid A, anchors LPS in the bacterial membrane, and is responsible for the majority of the biological effects of these potent molecules. Most of the time, lipid A moieties are linked to a core oligosaccharide, through an acidic deoxy sugar the 2-keto-3-deoxy-octulosonic acid (Kdo). The third moiety of LPS molecules, named the O-chain, is made of oligosaccharide repeating units extending outside the bacteria. These O-specific chain structures, being unique to a given bacterium, are at the origin of the serotyping, historically used to identify Gram-negative bacteria.

A certain number of important pathogens display LPS without O-chain, thus named lipooligosaccharide (LOS). Examples are *Bordetella pertussis*, *Neisseria meningitidis*, and *Haemophilus influenzae*.

LPS contributes to the outer membrane's integrity, it constitutes an efficient permeability barrier to antimicrobial compounds, and a protection against the complement-mediated lysis thanks to the length of its O-chains. A good example of such protection was described in *Shigella* (West *et al.*, 2005). Mutants showing Rough-type colonies on agar plates do not have O-chains in their LPS, they are more sensitive to the complement system and to antibiotics, than bacteria of the Smooth-type. The shortest LPS mutant structure is the deep-rough LPS type, it corresponds to a lipid A moiety

☆ Contribution to the Topical Issue "Microbiota, Nutrition and Lipids: consequences on Health".
☆☆ This paper is dedicated to the memory of Dr Yvon LeBeyec, a dear friend and colleague.
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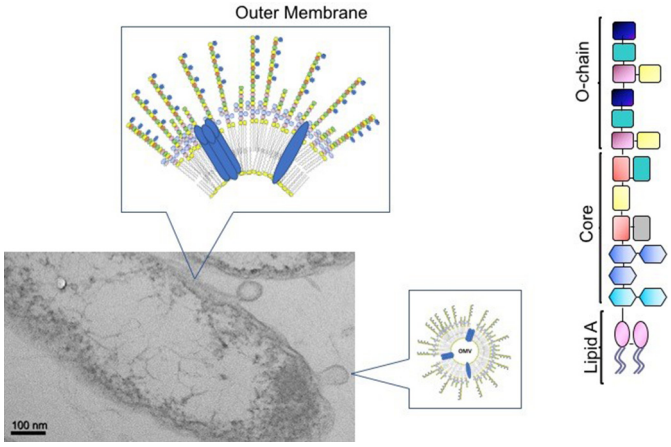

Fig. 1. Electron microscopy of a *Ralstonia pickettii* bacterium and schematic representation of the outer membrane of Gram-negative bacteria, outer membrane vesicles (OMV), and a LPS molecular species.

plus at least one Kdo molecule. Exceptions to the rule exist, and a mutant devoid of LPS has been described with *N. meningitidis* by insertional gene inactivation of the first step of LPS biosynthesis (Steeghs *et al.*, 2001, 1998).

The bacterial viability was only possible when a capsular polysaccharide was present, and differences were observed in the phospholipids composition (Steeghs *et al.*, 2001). Another example was described with *Moraxella catarrhalis* (Peng *et al.*, 2005). Apart from these exceptions, in the large majority of cases, LPS is essential for bacterial survival like in case of *E. coli*.

2 Lipopolysaccharide structures/function relationships, and bacterial detection

In 1899, R. Pfeiffer gave the name "endotoxins" by opposition to exotoxins because such toxins were not released by the bacteria-like proteins liberated in the course of the infectious process (Rietschel and Cavaillon, 2003). However, some LPS molecules get liberated during bacterial reproduction by fission of single cells, or when bacteria are killed, for example by use of antibiotics. If LPS get released in large amounts into the host blood, they can cause a massive inflammatory response and septic shock usually leading to main failure of most organs and death. When, on the contrary, LPS are present in small amounts, they stimulate the host-immune response and can boost the immune system. These two drastically opposed reactions explain the constant interest of scientists for these amazing molecules.

2.1 Structural analysis

The main characteristic of LPS molecules is their capability to display a unique structure for a given bacterium. Being highly heterogeneous molecules in each of their three moieties, they necessitate complex and thorough analyses for their characterization. Such analyses are performed mainly by mass spectrometry (MS), nuclear magnetic resonance (NMR), liquid and gas chromatography-mass spectrometry (LC-MS and GC-MS), sodium dodecyl sulfate electrophoresis (SDS-PAGE) and chemical analyses. We keep innovating in LPS structural analyses and already described major methods in the past decades, as summarized in Novikov *et al.* (2017).

2.2 Lipid A structures

One of the most described LPS structure is that of *E. coli* because it originates from an extensively studied bacterium. This is why non-specialists often extrapolate this structure as to "the" general one. To illustrate the real high variability of these molecules, some examples of lipid A structures varying among genera, and even in a single genus, are displayed in Figure 2. In most Enterobacteriacea, the lipid A structure corresponds to a bis-phosphorylated β-1-6 glucosamine disaccharide, carrying fatty acids (FA) in ester- and amide-linkages (Fig. 2).

Variability in the structure resides in the length of the FA aliphatic chains, as well as in the number of FA present on the disaccharide, varying from 2 to 9. The phosphate groups are most often substituting glucosamine I (GlcN I) at C-1, and GlcN II at C4'. They are also at the origin of some variability, as they can be either present or absent, and substituted, or not, by amino-sugars (AraN, GlcN, GalN) or phosphoethanolamine (PEA), and other residues (like methyl-) often referred to as "decorations" (Novikov *et al.*, 2014).

2.3 Unusual lipid A structures

While in the most widely described structures, lipid A is made of a GlcN disaccharide, other lipid A disaccharides were described with a 2,3-diamino-2,3-dideoxy-D-glucose (DAG) backbone, or with mixed compositions (Fig. 3).

A diaminoglucose disaccharide was found in structures such as those of *Brucella*, *Legionella*, *Rhizobia* and *Ochrobactrum* (Bundle *et al.*, 1987; Caroff *et al.*, 1984b; Lapaque *et al.*, 2006; Mayer *et al.*, 1989; Qureshi *et al.*, 1994; Sonesson *et al.*, 1989; Velasco *et al.*, 1998; Zähringer *et al.*, 1995). In some other cases, the disaccharide is a mixed structure with backbones composed of one DAG and one GlcN residues.

Campylobacter's lipid As were described as very complex structures as they contain a mixture of three types of disaccharide backbones: the classical di-GlcN, the di-DAG backbone and a mixture of GlcN-DAG disaccharide (Moran *et al.*, 1991).

Other structures with lipids A containing GlcN or DAG disaccharide backbones were also described as being devoid of phosphate groups, like in the genus *Rhodopseudomonas*, growing in soil and aquatic media (Holst *et al.*, 1983; Okamura *et al.*, 2009).

The phototropic bacterium *Rhodospirillum fulvum*, with a classical lipid A di-GlcN backbone carries an unusual heptose residue at C-4' and a galactosamine uronic (GalA) residue at C-1 (Rau *et al.*, 1995).

Rhizobia lipid A have been described as non-phosphorylated lipids A carrying long-chain FA and their GlcN 1 residue is replaced by an acylated 2-aminogluconate (Mayer *et al.*, 1989).

2.4 Cores structures

The core oligosaccharide is built up of a tens of monosaccharides arranged both in linear and branched structures. It consists of two distinct regions: the "inner core" proximal to the lipid A moiety, and the "outer core" to which

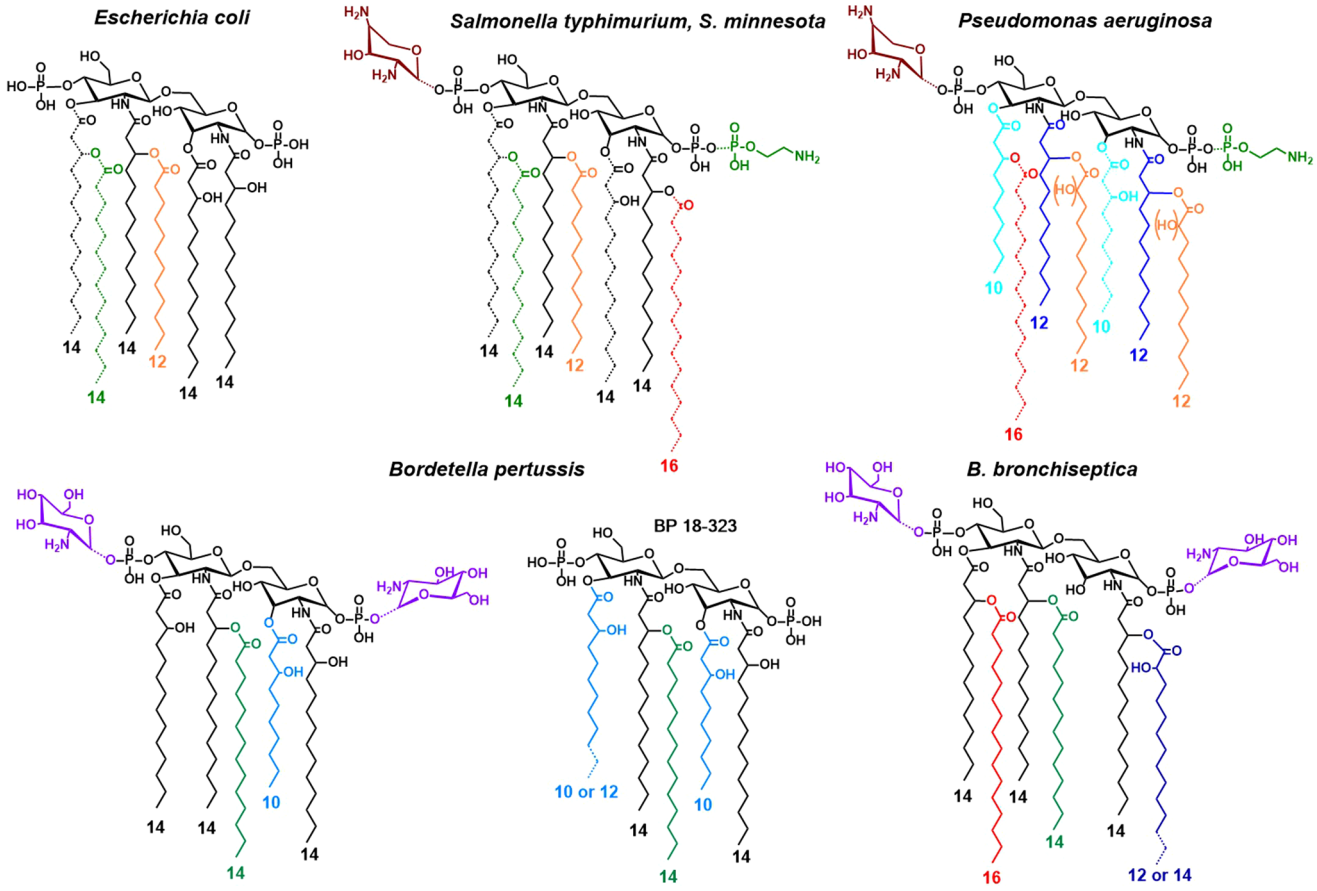

Fig. 2. Examples of lipid A structures.

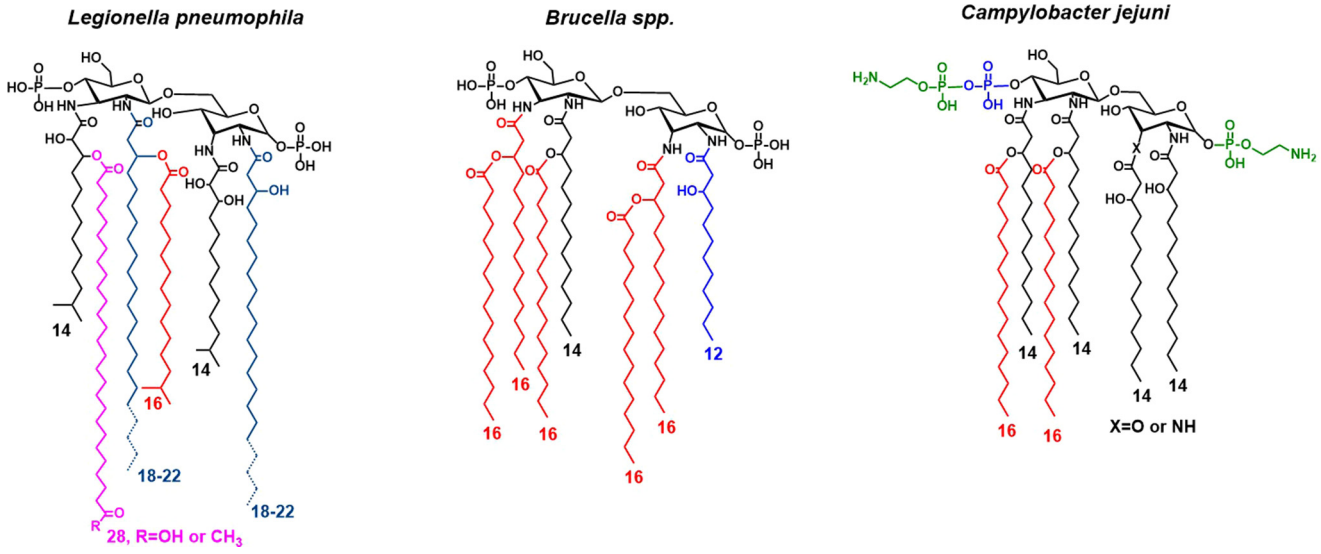

Fig. 3. Examples of unusual lipid A structures.

the O-antigen can be linked (Holst, 2011). The inner-core structures usually represent the more conserved part of the core. In Enterobacteriaceae, cores are made of Kdo and L-glycero-D-manno-heptose residues (Hep). The first Kdo linked to the *O*-6' position of lipid A through an α-ketosidic linkage is named Kdo-I, it can be substituted by one or two other Kdo residues (Kdo-II, and Kdo-III). Kdo-I is glycosylated at the *O*-4 position by Hep, the first one designated as Hep-I, it can also be decorated by P, or PEA or by one or two other Hep, or another sugar making the inner-core structure.

The enterobacterial "outer core" consists of an oligosaccharide of up to 6 sugars with Glc, Gal, GlcN, all in pyranose form, and in general displaying the α-anomeric configuration (Caroff and Karibian, 2003). Figure 4 shows a few core examples like that of *E. coli* R3 core type, which is present in the serogroups O:157, O:111 and O:26 (Amor *et al.*, 2000; Currie and Poxton, 1999).

The major modifications of *E. coli* core structures are most often made of nonstoichiometric additional Kdo, phosphate, PEA, rhamnose, and GlcN residues. It was shown that with PEA addition, originating from phosphatidylethanolamine, the bacterium becomes non-sensitive to antibiotics like polymixin and detergents (Yethon *et al.*, 1998). Addition of positively charged groups like 4-AraN and PEA to the lipid A neutralizes the negative charges of its phosphate groups and prevents recognition and attachment of polymixin, a cationic antibiotic molecule.

Another gene specific to PEA addition was shown to be involved in Ca^{++} hypersensitivity (Reynolds *et al.*, 2005). The addition of PEA induced by high Ca^{++} concentrations, protects bacteria from damages resulting from these high Ca^{++} concentrations. The molecular mechanism conferring this protection is not clear, but might be related to the capacity of PPEA to chelate multicharged Ca^{++} ions.

The *Pseudomonas aeruginosa* core contains non-carbohydrate substituents such as P, PPEA, acetyl, carbamoyl residues and amino acids. *P. aeruginosa* also synthesizes two types of cores, one being caped and covalently linked to the O-chains, the second uncapped and devoid of O-chain (Lau *et al.*, 2009).

2.5 Lipooligosaccharide core structures

As already mentioned, a number of highly virulent pathogens including *B. pertussis*, *H. influenzae*, *N. meningitidis*, and *Campylobacter jejuni* do not display an O-chain structure. Therefore, it is their complex and highly decorated core structures that are responsible for the bacterial specificity. The structure of *H. influenzae* and *B. pertussis* cores are depicted in Figure 4. The first one can be decorated, as indicated, by a glycine amino-acid substituent in various positions. This core also often carries phosphorylcholine residues (Post *et al.*, 2016; Schweda *et al.*, 2007). The *B. pertussis* core is composed of a first nonasaccharide core structure with unusual non-acylated amino groups at the two GlcNs and GalNA, and a distal trisaccharide originating from a different biosynthetic process. Effectively, this trisaccharide carries acetyl groups on each of the 4 amino groups of the highly immunogenic distal trisaccharide (Allen *et al.*, 1998).

LOS vary with environment and culture conditions, their cores are often longer than usual cores and can extend up to 15 sugars. In non-typable *H. influenzae* (NTHi), a distal Kdo or Neu5Ac could be added to the distal part of the structure on *N*-acetyllactosamine (Apicella *et al.*, 2018).

2.6 O-chains structures

O-chains are known as the most variable structures in LPS molecules, and one cannot find two identical LPS structures for two different bacteria. If a very little number of identical O-chain structures were described, like with *B. abortus* and *Yersinia enterocolitica* O:9 (Caroff *et al.*, 1984a, 1984b), their lipid A moiety and core structures remain different which makes different LPS structures in the end. In the great majority of cases, the O-chain structures can be compared to fingerprints, due to their uniqueness.

The repeating units of O-chains are typically made of oligosaccharides of 1 to 8 sugars. They are linear or branched structures and contain substituents like phosphate groups, O-acetyl groups, glycerol, ribitol, etc. The wide range of linkages between two consecutive sugars explains the high diversity of these structures. If there is only one possibility to form an amino-acid homodimer, there are 11 possibilities when it comes to a hexose disaccharide, due to the number of hydroxyl groups and stereo specificity of sugars, as well as to their anomeric linkage. Therefore, if there is again only one possibility for assembling a homotrimer amino-acid, there are 176 possibilities when it comes to hexoses, and 6 possibilities for an hetero-trimer amino-acid, compared to 1056 possibilities with three different hexoses. In fact, the number of existing combinations is reduced, due to specificity of biosynthetic enzymes. It remains however by far higher, compared to any other type of biomolecules. The number of combinations when it comes to sugars explain the complexity and diversity of polysaccharide structures.

Some examples of O-chain structures are displayed on Figure 5.

3 Lipopolysaccharides biosynthesis

LPS biosynthetic processes have well been described by Raetz *et al.* and for *E. coli* they are known as the "Raetz pathway" (Raetz, 1990; Raetz and Whitfield, 2002). The lipid A biosynthetic enzymes were also well described by Trent *et al.* in 2004 (Trent, 2004; Trent *et al.*, 2006). The complete LPS biosynthesis pathway starts with the lipid A-Kdo2 moiety molecule, which is first synthesized at the surface of the cytoplasmic membrane. The other core sugars are added to Kdo, into the inner membrane, before the lipid A-core molecules get flipped into the periplasmic space of the cytoplasmic membrane thanks to MsbA. The O-antigen is then synthesized by cytoplasmic membrane-associated enzyme complexes using C55 undecaprenyl-P as an acceptor for chain assembly, and is itself flipped into the periplasmic space of the membrane by one of the three following systems embedded into the outer membrane: Wzy dependent, ABC transporter dependent and synthase dependent (Valvano *et al.*, 2011).

LPS molecules display highly diverse structures and high heterogeneity after their biosynthesis at the level of the cytoplasmic membrane surface due to a large number of enzymatic steps which can be consecutive or parallel,

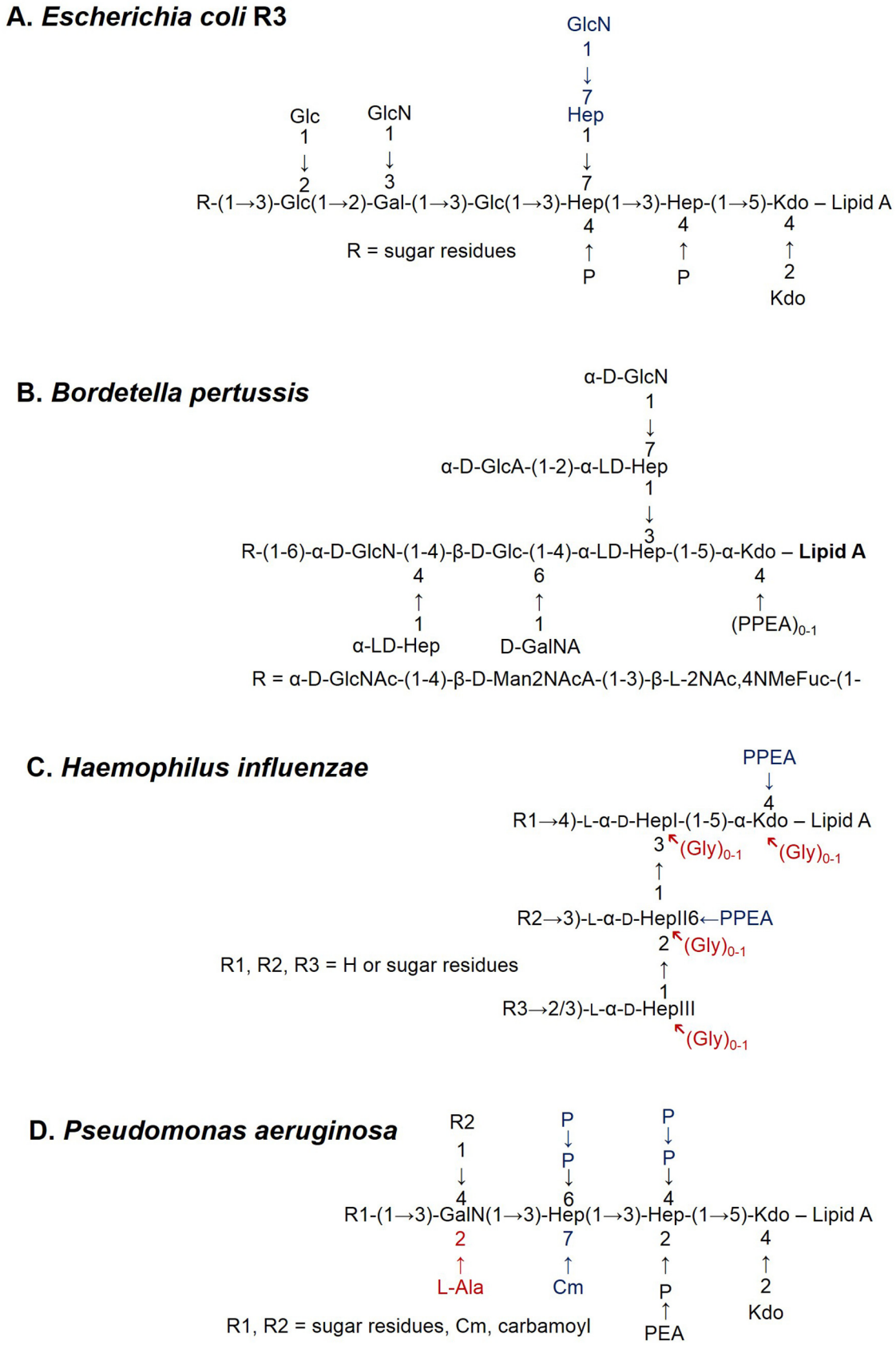

Fig. 4. Examples of core structures.

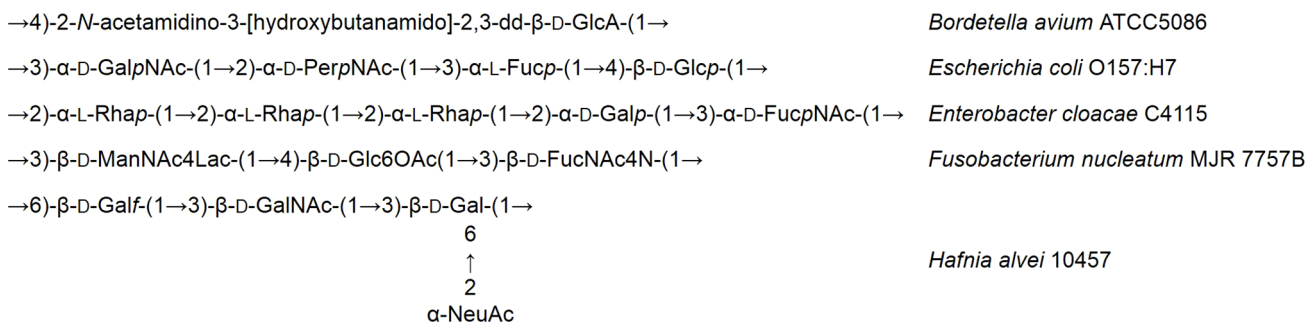

Fig. 5. Examples of O-chain structures.

independent or competing. The late stages of the biosynthesis, known as post-translational biosynthesis modifications, are adding lots of heterogeneity to these already heterogeneous molecules. These modifications are operated by enzymes often regulated *via* two component regulators (*e.g.* PhoP-PhoQ) in response to stress (Delgado *et al.*, 2006; Groisman, 2001; Gunn, 2001; Richards *et al.*, 2010). This provides to bacteria a powerful and flexible mechanism of adaptation to the environment, as well as to modifications of the growth conditions when it comes to planktonic cultures. Such modifications are for example the addition of FA like 16:0, or the removal of some of the FA added during the constitutive biosynthesis process in the cytoplasm. These modifications take place when the entire LPS molecule is already inserted in the outer membrane and the added palmitate residues originate from other bacterial components such as phospholipids (PPL) or lipoproteins. Other late-steps structural modifications consist in addition of AraN, GlcN, GalN or PEA on the phosphate groups of lipid A. These "decorations" take place in the periplasmic space during transport of LPS molecules from the outer surface of the cytoplasmic membrane to the inner surface of the outer membrane (Shah *et al.*, 2013).

Multiple structural lipid A modifications in the same pathogens, induced by changing of bacterial lifestyle from planktonic culture to biofilm conditions were demonstrated by Chalabaev *et al.* (2014) and Ciornei *et al.* (2010). These main examples show how imprudent it could be to trust laboratory growth conditions, for representing natural highly variable growth conditions, especially at the level of LPS structures easily modified according to niches and environment.

4 Lipopolysaccharides and metabolic diseases

A lot of attention has recently been given to the intestinal microbiome and its impact on health (Garidou *et al.*, 2015; Kamada *et al.*, 2013; Zhang-Sun *et al.*, 2015). There are 100 trillions microorganisms in the microbiota, namely bacteria, viruses, archaea, and eukaryotes (Thursby and Juge, 2017). They participate to the immune system maturation, help against pathogens, and secrete useful vitamins.

It was shown that an increase in Gram-negative bacteria occurred in obese patients as well as in rich-diet mouse models. An increase in low-inflammatory LPS amount in the blood of patients with such metabolic diseases could explain the observed reaction leading to impact the insulin receptor (Cani *et al.*, 2007).

The presence of *Ralstonia* has been demonstrated to increase in patients with metabolic diseases, we thus characterized the lipid A structures of three different species from this genus (Zhang-Sun *et al.*, 2018). The hypo-acylated penta-acyl *Ralstonia picketti* lipid A structure, together with the decoration with AraN on both phosphate groups, was characterized. Both substitution with AraN and under-acylation are known to favor the liberation of blebs, also called outer membrane vesicles (OMV), from the bacterial membrane (Elhenawy *et al.*, 2016). We also showed by electron microscopy (Fig. 6) that the thickness of the bacterial membrane varied with LPS structures, and that the short LPS of *R. pickettii*, together with its lipid A under-acylation and phosphate groups decorations was generating more blebs from its thin membrane. These blebs cross the intestinal membrane more easily than bacteria can do, and deliver more LPS to the blood. However, the characterized structure was shown to induce low levels of cytokines, if any, as shown in Figure 6, which was also in adequation with the low-grade inflammation level described in obese patients. This represents a new example of low inflammatory capacities of LPS originating from the intestinal microbiome, explaining that these LPS were not as toxic and dangerous as the *E. coli* type classical structures. Other examples were published by d'Hennezel *et al.* (2017) concerning the deacylated structure of bacteroidales LPS, originating from the human microbiome and their silenced Toll-like signaling capacity, explaining host-tolerance to the gut LPS microbiome.

5 Lipopolysaccharides detection methods

5.1 Limulus amoebocyte lysate

The capacity to detect endotoxins in drugs is essential, as these molecules, when toxic, are known to lead to septic shock and death when present in the blood at low concentrations. The international pharmacopea have imposed the limulus amoebocyte lysate (LAL) tests for LPS detection. *Limulus polyphemus*, the horseshoe crab lymph, is used to detect femtograms of LPS in pharmaceuticals, parenteral, implants... In this crab, as in other arthropods and mollusks, the oxygen transport relies on a copper-based pigment, hemocyanin giving a blue color to the lymph. The presence of LPS results in lymph coagulation and different tests have been set up since the 1970s. LAL is recognized as one of the most sensitive detection method, but other gel-clot, turbidimetric and chromogenic assays have been developed since the first test

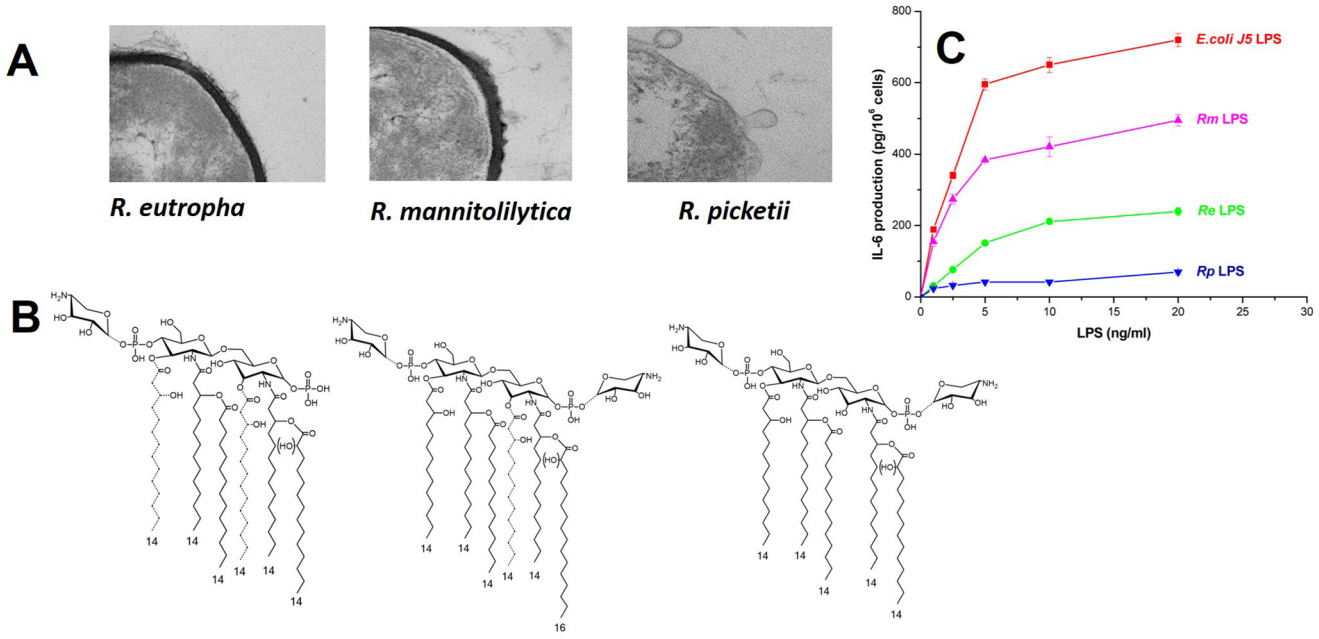

Fig. 6. (A) Electron microscopy showing the membrane thickness of three different *Ralstonia* species together with (B) their lipid A structures (Re: *R. eutropha*; Rm: *R. mannitolilytica*; Rp: *R. pickettii)*, and (C) comparison of their LPS IL-6 inducing capacities.

was developed. The method used in pharmaceuticals was also used to detect the presence of heat stable endotoxin (LPS) in powdered infant milk (Townsend *et al.*, 2007).

However, some glucans, proteins, blood factors and exotoxins can interfere with the test, and pH temperature, and ionic strength can influence the detection. A phenomenon called low endotoxin recovery (LER) disrupts LPS detection, it is thoroughly described in "Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems | SpringerLink" (n.d.).

In order to protect the horseshoe crab, an alternative has been introduced with use of factor C, a lysate protein produced to replace the crab lymph. Its sensitivity reaches 0.001 EU/ml. It also allows overcoming interferences of certain factors but does not seem to be a panacea.

5.2 Liquid chromatography-mass spectromtry (LC-MS2)

Detection of FA markers, the famous 3-OH FA, specific to Gram-negative bacteria, has been developed to estimate an equivalent amount of endotoxin, based on *E. coli* used as a standard. It is less sensitive than LAL, but less prone to interferences and can be used for detection in blood.

5.3 Serological and structural detection

Bacterial detection kits can be prepared for Elisa tests as described in Sippel *et al.* (1987). Such kits or strips can also be used to detect the presence of food borne bacteria in stocks of crops (Bruno, 2014).

Due to their main characteristics as unique structures representing a given species, LPS are precious tools for bacterial detection and characterization. Mass spectrometry detection methods can be used to characterize specific LPS structures. Lipid A structures are characteristic of a given bacterial genus, and can be used as phylogenic tools. On the other hand, the O-chain structures are specific of a single bacterial species and are thus useful to detect a specific bacterium.

5.3.1 Antibodies

LPS can be used to prepare antibodies, used for bacterial detection with a high specificity and sensitivity. The use of LPS antibodies has been performed for defining bacterial serology long before the O-chain structures could be characterized. Anne-Marie Staub, from the Pasteur Institute, established the polysaccharide nature of bacterial O-antigens (Staub and Tinelli, 1956). In collaboration with the German researchers, Otto Westphal and Otto Lüderitz, they demonstrated that repeating units of one to 10 sugars formed the LPS species-specific O-antigens (Staub *et al.*, 1959).

Numerous tests based on LPS detection to assess bacterial presence were set up in the past decades. They were used in Elisa and strip tests (Wang *et al.*, 2011).

5.3.2 Matrix assisted laser desorption mass spectrometry (MALDI-MS)

LPS detection through its lipid A MALDI-MS analysis was recently improved in terms of sensitivity. It can be used at the same time to characterize LPS, and to get information on the bacterial genera.

We have ourselves defined a good level of sensitivity by detecting in MALDI-MS molecular ions corresponding to the *E. coli* lipid A moiety at *m/z* 1797, using serial dilutions of bacterial cultures and not dilution of a final concentrated lipid A extract. This method can be used to characterize the origin of the detected LPS, as well as the capacity of the bacterium to

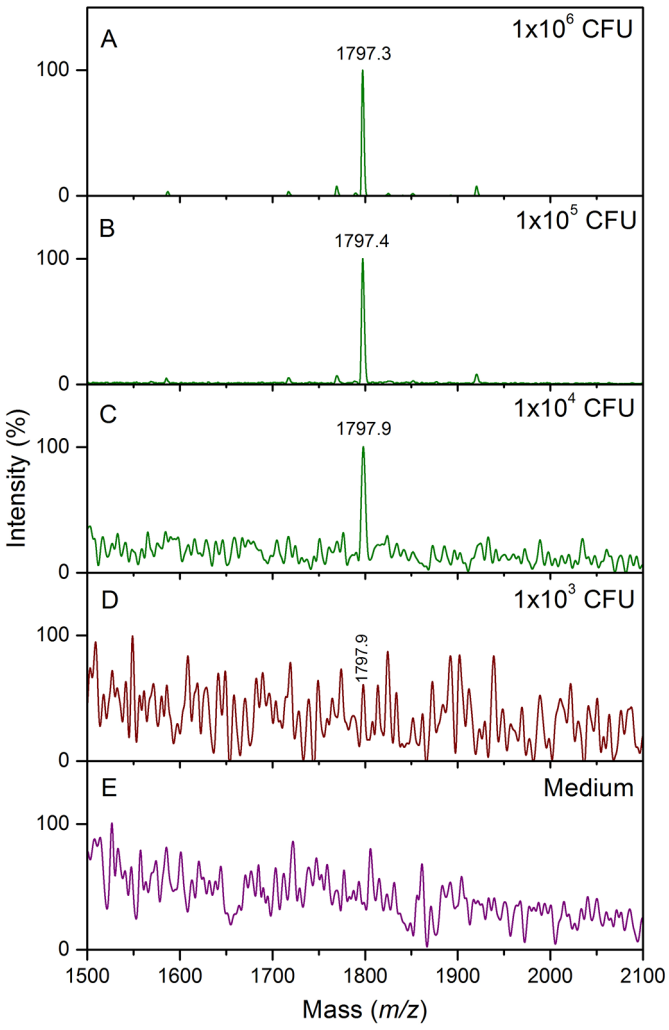

Fig. 7. Maldi-MS detection of a *E. coli* lipid A characteristic ion at *m/z* 1797 in a 1 ml bacterial culture sample containing a total number of bacterial cells as low as 1×10^3–1×10^4CFU.

resist to polymixin according to the presence or absence of decorations on the lipid A phosphate groups.

The results are presented in Figure 7 and show that a characteristic peak can be obtained from a 1 ml bacterial culture sample containing a total number of bacterial cells as low as 1×10^3–1×10^4CFU. Comparable experiments published by other authors gave detection limit of 1×10^5CFU (Leung *et al.*, 2017).

All three described methods should be considered when LPS detection is vital, like in the medical and pharmaceutical domains. When interferences occur, one method can compensate the others, and use of at least two complementary methods is highly recommended.

6 Conclusion

LPS are amazing ubiquitous molecules, they are tracked down to avoid their presence in pharmaceuticals, or passage to the blood during infection, potentially leading to septic shock. On the other hand, the capacity of these molecules to stimulate the immune systems at low doses, or by use of detoxified molecules, opens a broad field of applications in the domain of vaccination and immunotherapy. If genomic studies helped understanding the biosynthetic pathways leading to LPS structures, the post-translational modifications happening after LPS transfer to the external membrane is difficult to predict and necessitates complex structural analysis methods and tools. A unique LPS structure corresponding to a given species or even a strain, the number of already characterized structures is therefore limited. The influence of LPS molecules, and their structures, in the human intestinal microbiome is a good example of the importance of these key molecules on health in recent developments. According to the current incapability to grow most existing bacteria, major discoveries, and developments, are still expected in the LPS domain.

Conflicts of interest. The authors declare that they have no conflicts of interest in relation to this article.

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