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Remodelling of the Gram-negative bacterial Kdo_2-lipid A and its functional implications

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Abstract

The lipopolysaccharide (LPS) is a characteristic molecule of the outer leaflet of the Gram-negative bacterial outer membrane, which consists of lipid A, core oligosaccharide, and O antigen. The lipid A is embedded in outer membrane and provides an efficient permeability barrier, which is particularly important to reduce the permeability of antibiotics, toxic cationic metals, and antimicrobial peptides. LPS, an important modulator of innate immune responses ranging from localized inflammation to disseminated sepsis, displays a high level of structural and functional heterogeneity, which arise due to regulated differences in the acylation of the lipid A and the incorporation of non-stoichiometric modifications in lipid A and the core oligosaccharide. This review focuses on the current mechanistic understanding of the synthesis and assembly of the lipid A molecule and its most salient non-stoichiometric modifications.

Lipopolysaccharide (LPS) is a unique surface molecule found on the outer leaflet of the Gram-negative bacterial outer membrane [1]. LPS consists of three structural components: lipid A, core oligosaccharide (core), and O-specific polysaccharide or O antigen [2]. The O antigen is not present in all bacteria. The lipid A moiety, referred to as Kdo_2-lipid A (see below), is embedded in the outer membrane, and provides an effective permeability barrier. This barrier is particularly important to reduce the permeability of antibiotics, toxic cationic metals, and antimicrobial peptides [1].

LPS is an important modulator of innate immune responses. During infection, LPS, and especially Kdo_2-lipid A, is recognized by the Toll-like receptor 4 (TLR4) in complex with myeloid differentiation factor 2 (MD2); this complex is on the surface of many cell types including macrophages and dendritic cells. LPS recognition stimulates signalling pathways that lead to the production of proinflammatory cytokines and Type I interferons. These innate immune responses range from localized inflammation to disseminated sepsis [3] Additionally, mammalian cells can recognise LPS in the cytoplasm in a TLR4/MD2-independent manner, which involves direct recognition by noncanonical inflammasome caspases 4/5 in humans and 11 in mice [4–6]. The core and the O antigen also provide additional protection to bacteria by adding to the barrier function and imparting resistance to phagocytosis and killing by complement, respectively [7, 8].

LPS biosynthesis, export, and assembly require multiple enzymes and structural proteins encoded by more than fifty genes [2]. *Escherichia coli* synthetizes lipid A, a β–1,6-disaccharide of glucosamine that is phosphorylated and substituted with acyl chains. Lipid A is glycosylated at the 6′-position with two residues of 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo); the inner Kdo serves as the point of attachment for the rest of the core. Both the lipid A and core are assembled on the cytoplasmic side of the inner membrane and translocated across the inner membrane by the ABC transporter MsbA [2]. The Kdo_2-lipid A is the minimal LPS component required for the cell viability of most Gram-negative microbes. Consequently, the genes encoding the proteins responsible for Kdo_2-lipid A biosynthesis and transport are essential for bacterial growth and replication; mutations in these genes dramatically compromise the integrity of the outer membrane.

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Abbreviations: ACP, acyl-carrier protein; CMP-Kdo, cytidylyl-monophosphate-β-Kdo; GlcNAc, N-acetyl glucosamine; Kdo, 3-deoxy-D-manno-oct-2-ulopyranosonic acid; KdoO, Kdo 3-hydroxylase; Ko, D-glycero-D-talo-oct-2-ulopyranosonic acid; L-Ara4N, L-4-amino-deoxy arabinose; LPS, lipopolysaccharide; Lpt, LPS transport pathway; MD2, myeloid differentiation factor 2; 3-OH-C10, R-3-hydroxydecanoate; 3-OH-C12, R-3-hydroxylaurate; 3-OH-C14:0, R-3-hydroxymyristate; TLR4, toll-like receptor 4; UDP-GlcNAc, uridine diphosphate-N-acetyl glucosamine; Und-P, undecaprenyl phosphate; Und-PP, undecaprenyl pyrophosphate.

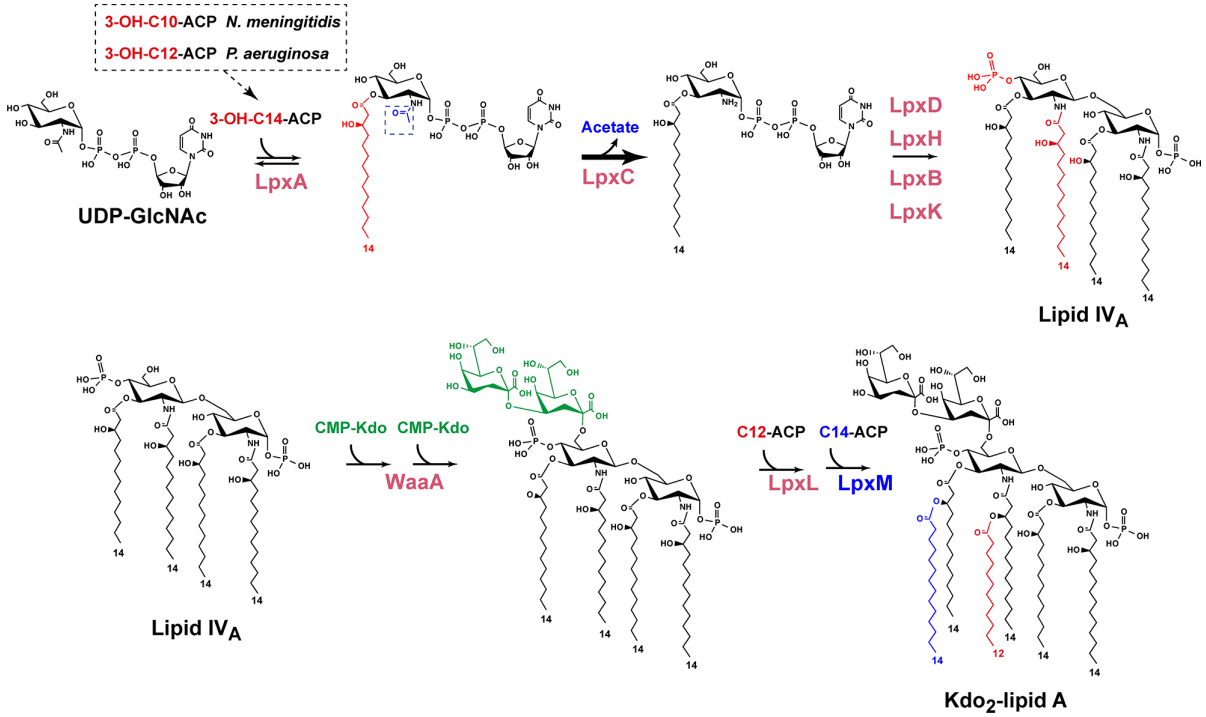

Fig. 1. Raetz pathway for Kdo_2-lipid A synthesis. The figure illustrates the most salient steps of the Raetz pathway [2, 21]. 3-OH-C10, R-3-hydroxydecanoate; 3-OH-C12, R-3-hydroxylaurate; 3-OH-C14:0, R-3-hydroxymyristate; ACP, acyl-carrier protein; CMP-Kdo, cytidylyl-monophosphate-β-Kdo; C12 and C14 denote C12:0 (lauroyl) and C14:0 (myristoyl) acyl groups, respectively.

The O antigen is separately assembled onto a C55-phosphopolyisoprenol, undecaprenyl phosphate (Und-P), forming an Und-PP-linked saccharide. This precursor is also translocated to the periplasmic side of the cell membrane by various well established mechanisms [2, 9]. The O antigen is finally ligated to the lipid A-core through a glycosyltransfer reaction that occurs on the periplasmic face of the inner membrane, which results in the release of Und-PP [10]. Released Und-PP is recycled into Und-P by a poorly characterized but presumably conserved pathway that involves the hydrolysis of the terminal phosphate [11, 12]. Newly formed LPS is then shuttled across the periplasm by a complex multiprotein pathway that spans the inner and the outer membrane, designated LPS transport pathway (Lpt), which is also responsible for the insertion of LPS into the outer leaflet of the outer membrane [13–15].

The O antigen displays great variability due to differences in the sugar composition, stereochemistry of the glycosyl bonds between sugars and non-stoichiometric modifications. While generally more conserved across bacteria, the Kdo_2-lipid A-core displays structural and functional heterogeneity, which arise due to regulated differences in the acylation of the lipid A and the incorporation of non-stoichiometric modifications in lipid A and the core, especially in the proximal region of the core including Kdo and heptose residues (classically referred to as inner core). This review summarises the current mechanistic understanding of the synthesis and assembly of the Kdo_2-lipid A molecule and its most salient non-stoichiometric modifications. Complementary information and additional details on various aspects of LPS assembly, regulation, and export to the outer membrane are reviewed elsewhere [7, 8, 16–20].

THE RAETZ PATHWAY OF KDO_2-LIPID A BIOSYNTHESIS

The Kdo_2-lipid A biosynthesis follows a canonical pathway known as the Raetz pathway, which consists of nine enzymatic reactions conserved across almost all Gram-negative bacteria (Fig. 1) [21]. All these reactions occur in the bacterial cytoplasm, involving soluble and membrane-associated enzymes.

The Raetz pathway starts with the ester linkage of an acyl chain to the 3-OH group of uridine diphosphate-*N*-acetyl glucosamine (UDP-GlcNAc), a reaction catalysed by LpxA. An acyl carrier protein (acyl-ACP) donates the acyl chain and is an obligate substrate for this reaction [22]. LpxA discriminates the length of the acyl chain attached to acyl-ACP [23], demonstrating the protein has a 'hydrocarbon ruler' [24, 25]. LpxA homologues differ in the preferred acyl chain length. In *E. coli*, the preferred hydrocarbon lipid is R-3-hydroxy myristate (3-OH-C14:O), but in *Pseudomonas aeruginosa* and *Neisseria meningitidis* LpxA

proteins are specific for R-3-hydroxy decanoate (3-OH-C10:0) [23] and R-3 hydroxy laurate (3-OH-C12:0) [26], respectively (Fig. 1). In contrast, LpxA homologues from *Porphyromonas gingivalis* [27], *Bordetella bronchiseptica* and *Bordetella pertussis* [28] show relaxed specificity for the acyl chain length. LpxA proteins also differ in their specificity for the 3-OH on the UDP-GlcNAc acceptor [29–31].

The next step in Kdo_2-lipid A biosynthesis involves deacetylation of UDP-3-*O*-(acyl)-GlcNAc by LpxC (Fig. 1); this is a Zn^{2+}-dependent deacetylase catalysing the formation of UDP-3-*O*-(acyl)-glucosamine. This reaction has an unfavourable equilibrium constant associated with UDP-3-*O*-(acyl)-GlcNAc production; it is therefore the first committed step of lipid A biosynthesis [32]. This property, plus the lack of homology of LpxC to other deacetylases and the essential nature of the reaction to support bacterial growth, makes LpxC attractive for developing a new class of antibiotics [33]. LpxC cellular levels are regulated post-transcriptionally by the membrane bound ATP-dependent metalloprotease FtsH [20, 34]; this in turn controls production of Kdo_2-lipid A. The levels of LpxC are regulated according to growth rate: slow growth increases LpxC degradation rate while rapid growth stabilizes the protein [35]. FtsH also regulates in a similar manner the incorporation of Kdo to lipid A [36] by controlling the cellular levels of the Kdo transferase WaaA (see below). LpxC is delivered to FtsH by the adaptor protein YciM(LapB), which is an integral membrane protein with a cytoplasmic region containing tetratricopeptide repeat motifs and a rubredoxin-like domain [37–39]. Recent evidence from several groups uncovered another essential membrane protein, YejM(PbgA/LapC), which regulates the LapB/FtsH protease complex [40–43]. The current model of regulation is that under physiological conditions, YejM inhibits the activity of the LapB/FtsH complex, which in turn stabilizes LpxC, promoting LPS biosynthesis. Accumulation of LPS in the outer leaflet of the inner membrane reduces YejM activity, lifting the block on the LapB/FtsH protease complex, which results in increased degradation of LpxC and reduction in LPS biosynthesis [40–43]. Additional evidence shows that YejM and LapB physically interact [42, 43]. Therefore, YejM appears to be a sensor for LPS/phospholipids balance at the membrane by binding LPS and phospholipids or both, which would determine its ability to modulate the amount of LpxC [42].

The Raetz pathway continues with the transfer a second acyl chain to the newly generated amine group to form UDP-2,3-diacylglucosamine, a reaction catalysed by LpxD. The *E. coli* LpxD shares similar properties as LpxA, including a hydrocarbon ruler specific for R-3-hydroxymyristate and the requirement of acyl-ACP as the acyl donor [44, 45]. LpxD proteins in various bacteria differ in selectivity for the acyl-ACP substrate [45, 46]. LpxD is also subject to posttranscriptional regulation linking LPS production to nitrogen metabolism [47].

The phosphoanhydride bond of UDP-2,3-diacylglucosamine is subsequently hydrolysed by the pyrophosphatase LpxH, resulting in UMP and 2,3-diacylglucosamine-1-phosphate (lipid X) [48]. LpxB is a disaccharide synthase that performs the next step, resulting in the condensation of one molecule of UDP-2,3-diacylglucosamine with one molecule of lipid X [49]. Several Gram-negative bacteria lack LpxH homologues; they use instead a distinct UDP-2,3-diacylglucosamine pyrophosphatase termed LpxI [50]. The next step in Kdo_2-lipid A synthesis is the phosphorylation at the 4′-position of the tetra-acylated monophosphorylated intermediate producing lipid IV_A [51–53] (Fig. 1). This reaction is catalysed by the integral inner membrane kinase LpxK and requires ATP hydrolysis. Discovery of LpxK facilitated the production of highly pure radiolabelled lipid A substrates for *in vitro* assays to characterize lipid A biosynthesis and modification enzymes.

The addition of Kdo residues to the lipid IV_A is an obligate step prior to completion of the Kdo_2-lipid A molecule. This reaction involves the transfer of two Kdo molecules to the distal glucosamine of lipid IV_A by the bi-functional enzyme WaaA (KdtA) [54–56]. WaaA enzymes are highly specific for the donor substrate, cytidylyl-monophosphate-β-Kdo (CMP-Kdo; Fig. 1), but they tolerate lipid A acceptor molecules of different acylation lengths and disaccharide backbones [57–60]. However, WaaA catalysis strictly depends on the presence of a negatively charged phosphate group at position 4′ of the lipid A intermediate [57]; some WaaA homologues transfer three or four Kdo residues [61–63], while others are monofunctional and therefore can only transfer one Kdo residue [60, 64–67]. The comparison of monofunctional and bi-functional chimeric WaaA proteins from *E. coli* and *Haemophilus influenzae* revealed that the N-terminal half of each protein was critical for the differences in functionality [68].

In *E. coli* and *Salmonella enterica*, hexa-acylated lipid A species are produced by the action of two acyl transferases, LpxL and LpxM, which catalyse the addition of secondary acyl chains to the distal glucosamine and usually referred to as 'late' acyltransferases [54]. These reactions occur after the transfer of Kdo residues. LpxL and LpxM share strong amino acid sequence similarity and cannot function if the absence of Kdo. LpxL first transfers a lauroyl (C12:0) group to the 2′-position of Kdo_2-lipid IV_A, which is followed by the LpxM-catalysed addition of a myristoyl (C14:0) group to the 3′-position completing the formation of Kdo_2-lipid A (Fig. 1). These enzymes utilize acyl-ACPs as their preferred acyl donor, but they share no homology to LpxA and LpxD [69, 70]. The requirement for acyl-ACPs is not universal; for example, the *Vibrio cholerae* LpxL uses acyl-CoA with similar efficiency as acyl-ACP [64]. In bacteria with a single Kdo, like *V. cholerae* and *H. influenzae*, the attachment of a phosphate group to the Kdo sugar is a prerequisite for the subsequent addition of secondary acyl chains [64, 71].

The 'late' acyltransferases of the Raetz pathway outside enteric bacteria show more diversity. For example, *Chlamydia trachomatis* and *Burkholderia cenocepacia* contain a single homologue of the *E. coli* enzymes, and this agrees with the fact that these bacteria synthesize a penta-acylated lipid A containing a single secondary linked acyl chain. In contrast, the Epsilon proteobacterium *Helicobacter pylori*, forms hexa-acylated lipid A despite the absence of a protein homologous to LpxM in the members of this

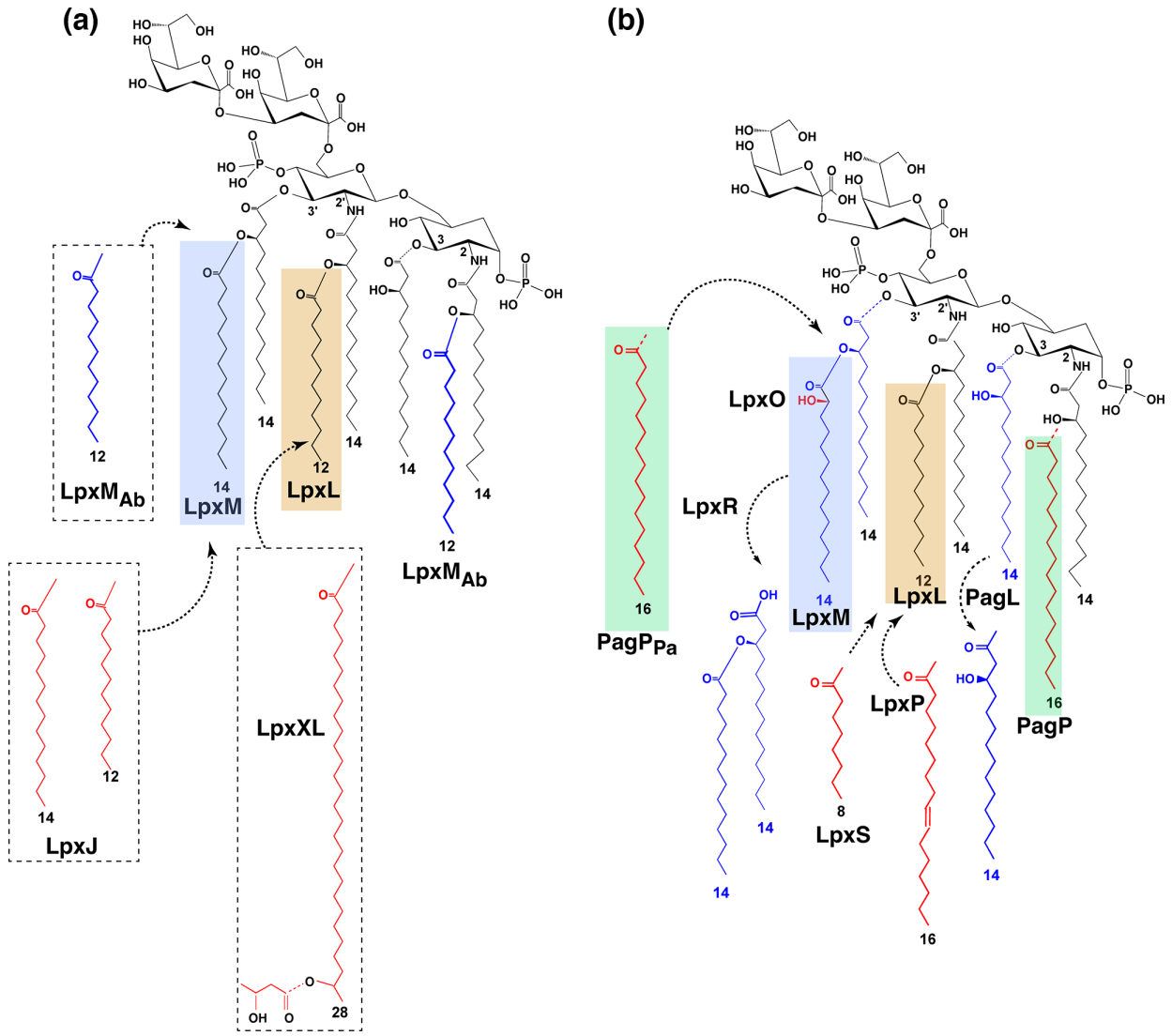

Fig. 2. Acyl chain modifications of Kdo_2-lipid A. a) The modifications of Kdo_2-lipid A by the canonical 'late' acyltransferases LpxM and LpxT from *E. coli* are indicated in the blue and ochre boxes, respectively; $LpxM_{Ab}$, LpxM protein from *Acinetobacter baumannii* that transfers lauroyl (C12:0) groups as secondary acyl chains at positions 2 and 3' [77]. LpxJ replaces LpxM in bacteria from the Epsilon group (e.g. *Helicobacter pylori*) and transfers either lauroyl (C12:0) or myristoyl (C14:0) groups as secondary acyl chains at position 3' [72]. b) The modifications by LpxM and LpxL are indicated as in (a); LpxP from *Escherichia coli* and LpxS from *Acinetobacter baumannii* [81] transfer palmitoleate (C16:1) and octanoate (C8:0) groups, respectively, replacing the function of LpxL under cold temperature; PagL and LpxR are deacetylases that remove Kdo_2-lipid A acyl chains as indicated; PagP adds a palmitate group as a secondary chain at position 2; $PagP_{Pa}$ is a divergent PagP protein from *Pseudomonas aeruginosa* [135] that transfers a palmitate group to position 3' as shown.

genus. *H. pylori* expresses LpxJ, a 'late' acyltransferase with an activity comparable to that of LpxM. As *E. coli* LpxM, LpxJ transfers a secondary acyl chain to the 3′-linked primary acyl chain of lipid A (Fig. 2a). LpxJ proteins share very little amino acid sequence with LpxM and form a large class of acyltransferases found in many bacteria that lack an *E. coli* LpxM homologue, suggesting that LpxJ participates in lipid A biosynthesis in place of an LpxM homologue [72]. In addition to the limited sequence similarity with LpxM, LpxJ has relaxed specificity and can complement an *E. coli lpxM* mutant since it catalyses the incorporation of C12:0 or C14:0 acyl chains. Further, *H. pylori* LpxJ and its homologues in *Campylobacter jejuni* and *Wolinella succinogenes* can act before the 2′ secondary acyltransferase, LpxL, and the WaaA Kdo transferase [72, 73]. Similarly, relaxed requirement of the Kdo residues for secondary acylation has been observed for *P. aeruginosa* [74, 75], and *N. meningitidis* [76] late acyl transferases.

Although in most cases LpxM catalyses the addition of one acyl chain, the *Acinetobacter baumannii* LpxM homologue transfers two C12:0 acyl chains at positions 3' and 2, respectively (Fig. 2a) [77]. This results in a PagP-independent mechanism (see the description of PagP function below) to synthesize hepta-acylated lipid A, which provides *A. baumannii* with the ability to resist killing

by antimicrobial peptides and tolerate desiccation [77]. In the plant endosymbionts *Rhizobium leguminosarum* and *Rhizobium etli*, incorporation of a secondary acyl chain requires the unique late acyltransferase LpxXL that transfers an unusually long acyl chain consisting of 28 carbons (Fig. 2a) [78], which may be important for the bacterial adaptation to life in the root nodule [79].

E. coli also expresses LpxP, a third late acyl transferase, but only under growth at low temperature. LpxP incorporates a palmitoleate (C16:1) in place of the canonical C12:0 added by LpxL to the primary acyl chain at 2' position (Fig. 2b). The introduction of an unsaturated acyl chain into the lipid A that may increase membrane fluidity at cold temperatures [80]. Alteration of the lipid composition is critical for maintaining membrane fluidity, permeability of the lipid bilayer, and protein function under diverse conditions. Several *Acinetobacter* species including *A. baumannii* have a cold-stress regulated acyltransferase named LpxS [81], which transfers an octanoate (C8:0) fatty acid in place of C12:0 at the 2′ position of lipid A (Fig. 2b). Expression of LpxS under cold conditions likely increases membrane fluidity and the effectiveness of the outer membrane permeability barrier. Therefore, different Gram-negative bacteria have evolved adaptive mechanisms to cold stress that involve the addition of either long unsaturated fatty acids (LpxP) or short-chain saturated fatty acids (LpxS) to the lipid A molecule.

REMODELLING KDO$_2$-LIPID A

The Kdo$_2$-lipid A molecule arising from the Raetz pathway acquires heterogeneity imparted non-stoichiometric modifications of its basic chemical structure. The enzymes catalysing these modification are typically expressed in response to environmental signals, allowing bacteria to adapt to adverse conditions including host defences and immune surveillance [16]. However, not all modifications appear to be regulated, as they might be essential for bacterial survival in specific niches within the host [82] or for survival in competition with other organisms that produce antibiotics and antimicrobial peptides [83]. Also, these modifications typically occur after the Kdo$_2$-lipid A (or the entire LPS molecule) has been transported to the periplasmic face of the inner membrane. Elucidating the genes and functions involved in remodelling lipid A-Kdo have helped develop combinatorial engineering approaches that can be exploited to generate a spectrum of immunostimulatory Kdo$_2$-lipid A chemical species for vaccine and therapeutics applications [84].

MODIFICATION OF THE LIPID A PHOSPHATE GROUPS

The lipid A phosphates at 1- and 4′ positions are prime targets for modification (Fig. 3). Presumably the ultimate role of these modifications is shielding the negative charges of the phosphates making lipid A 'blind' to recognition by antimicrobial peptides and other cationic molecules. In general, phosphates are either removed or covalently modified.

Removal of phosphates involves highly specific lipid A 1- (LpxE) and 4′-phosphate (LpxF) phosphatases, which belong to the phosphatidic acid-phosphatase superfamily (Fig. 3) [85–87]. Mutants unable to dephosphorylate lipid A become sensitive to antimicrobial peptides [88–91], are attenuated in infection models [90, 92–94], and result in lipid A species with altered TLR4-mediated responses [95], highlighting the biological importance of the 1- and 4′-phosphate groups of the lipid A.

A classical covalent modification of lipid A phosphates is the incorporation of L-4-amino-deoxy arabinose (L-Ara4N) by the ArnT transferase (Fig. 3) [96]. L-Ara4N is synthesized as an Und-P-linked precursor and is flipped to the periplasmic side of the membrane. ArnT utilizes Und-P-L-Ara4N as a donor, modifying lipid A on the periplasmic side of the inner membrane [97]. ArnT preferentially modifies the 4′-phosphate group and requires the presence of the secondary acyl chain at the 3′-position for optimal activity [82]. PmrA/PmrB and PhoP/PhoQ two-component regulatory systems regulate ArnT expression in *S. enterica* [98], but ArnT appears to be constitutively expressed in other bacteria [83]. The presence of L-Ara4N in the lipid A confers resistance to antimicrobial peptides [99], and *Salmonella* mutants unable to synthesize L-Ara4N-modified lipid A are also attenuated in virulence [100]. In *B. cenocepacia*, the modification of lipid A by L-Ara4N is absolutely essential for bacterial survival [101], as only L-Ara4N decorated lipid A is a substrate for the Lpt export pathway in this bacterium [83, 102]. This resulted in the first demonstration that lipid A Lpt export apparatus can discriminate substrates based on the chemical groups placed at position 1 and/or 4′ of the Kdo$_2$-lipid A instead of membrane embedded lipid A acyl chains, as previously thought. In *B. cenocepacia* and other bacteria, the second Kdo residue is D-glycero-D-*talo*-oct-2-ulopyranosonic acid (Ko) which is synthetised by a unique Kdo 3-hydroxylase (KdoO) (Fig. 3), which is present in species of *Burkholderia*, *Yersinia*, *Klebsiella*, *Legionella*, *Coxiella*, and *Ralstonia* [103]. This residue can also be L-Ara4N-modified, but it is unclear if this modification is ArnT-dependent (Fig. 3).

ArnT proteins are not only implicated in lipid A modifications with L-Ara4N, as it has been found that some ArnT homologues transfer glucosamine and galactosamine or mannose in *Bordetella* and *Francisella* species, respectively (Fig. 3) [104–106]. *Francisella* mutants unable to modify their lipid A with either galactosamine or mannose are attenuated in virulence [106], while the addition of glucosamines to the *B. pertussis* lipid A increases its ability to stimulate human TLR-4 activation and produce proinflammatory cytokines [107]. Further, *Rhizobium* has an ArnT-like enzyme that transfers galacturonic acid to the disaccharide backbone of lipid A at the 4′-position after LpxF cleaves the phosphate group [87]. The lipid A of two species of *Aquifex* has galacturonic acid at the 1- and 4′-positions [60, 108]. Therefore, ArnT proteins appear to form a family of glycosyltransferases with a range of specificities, which target the lipid A phosphates at positions 1 and 4′ (Fig. 2). Structural comparison of ArnT [109]

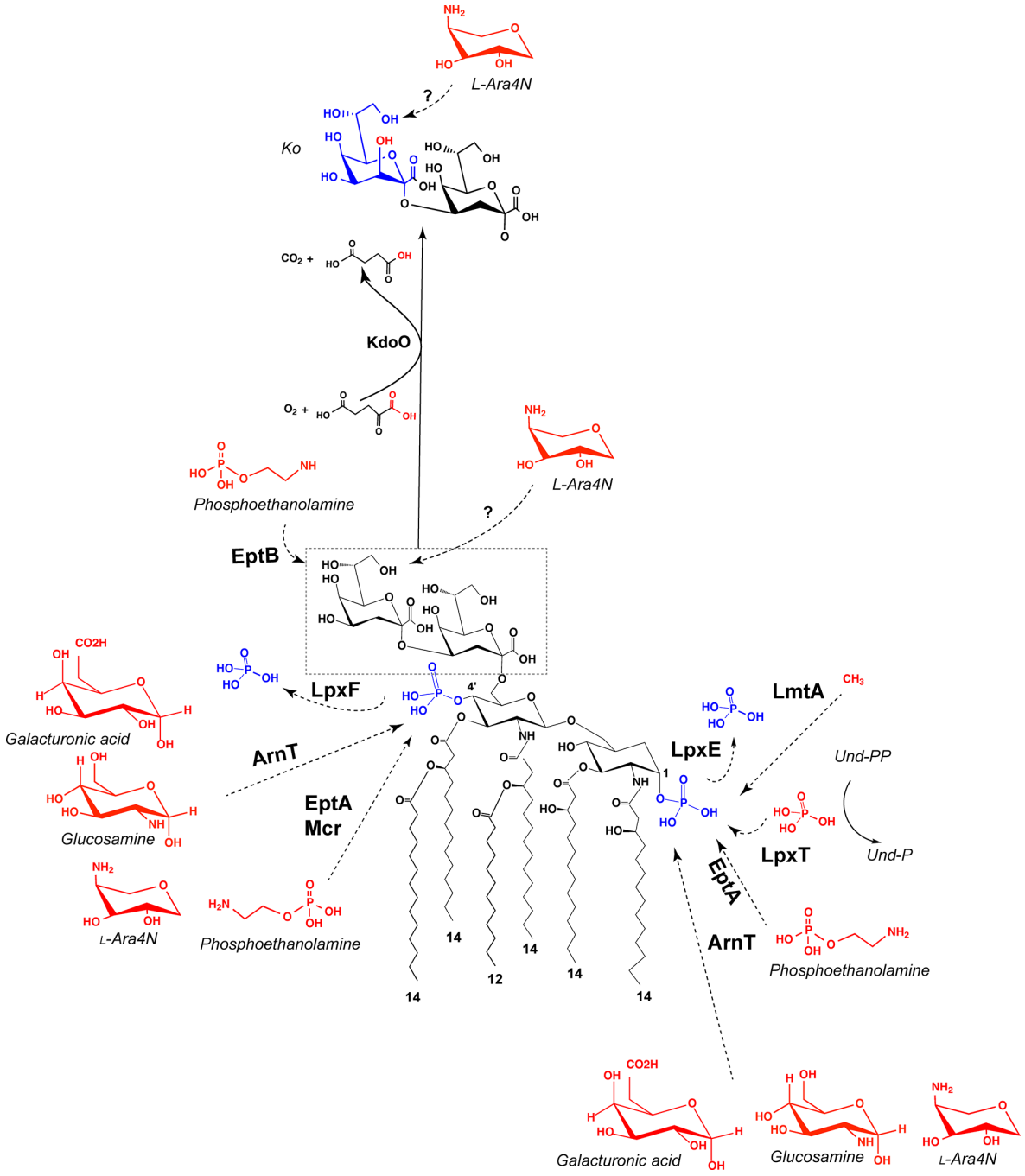

Fig. 3. Scheme showing modifications of Kdo_2-lipid A phosphate residues at positions 1 and 4' and most commons modifications in Kdo and Ko residues. The various molecules added or removed at positions 1 and 4' of Kdo_2-lipid A together with the names of the corresponding enzymes are shown; KdoO, Kdo 3-hydroxylase implicated in the synthesis of Ko; ? denotes that the enzyme modifying Ko with L-Ara4N is unknown.

and the *N*-oligosaccharyl transferase PglB involved in protein glycosylation [110], also performing catalysis at the periplasmic side of the membrane, suggests these enzymes may have an evolutionary link [111].

Another classical modification targeting Kdo_2-lipid A phosphate groups and contributing to antimicrobial peptide resistance is the addition of phosphoethanolamine (PEtn), a reaction catalysed by EptA (Fig. 3), which is also under the control of PmrAB [112, 113]. EptA is also found in *H. pylori* [89], *N. meningitidis* [114, 115], and *C. jejuni* [116]. EptA can transfer PEtn to either 1 or 4′ phosphates, but the enzyme preference can vary depending on the bacterial species and the presence or absence of additional modifications [113]. PEtn modification of Kdo_2-lipid A can also be mediated by a PEtn transferase encoded by the

mobilized colistin resistance (*mcr*) gene [117]. Molecular epidemiological studies have identified at least ten gene variants, *mcr*-1 to *mcr*-10, all of which encode structurally similar enzymes that share structural similarity with *N. meningitidis* EptA [117]. The *mcr* genes are flanked by insertion elements, which facilitate their mobility to different plasmids that in turn can be mobilizable to many different bacteria. This has greatly complicated the use of polymyxins as last-resort antibiotics for the treatment of infections caused by highly multidrug resistant bacteria and has rapidly raised as a serious public health concern worldwide [118].

The *E. coli* K-12 LpxT phosphorylates lipid A at the 1-position forming 1-diphosphate lipid A [12] (Fig. 3). This modification is present in about one-third of the lipid A molecules in the outer membrane, and the phosphate donor molecule for this reaction is undecaprenyl pyrophosphate (Und-PP) [12]. Und-PP is the carrier lipid for peptidoglycan, O antigen and other bacterial surface carbohydrate polymers, which is produced *de novo* and also recycled after releasing its cargo [11]. The release of Und-PP-linked polymers takes place in the periplasmic side of the inner membrane, where the catalytic site of LpxT is also located [119]. Therefore, LpxT links the biosynthesis of lipid A with the recycling with Und-PP and the assembly of other essential cell envelope structures. Although LpxT homologues generally phosphorylate at the 1-position, the *P. aeruginosa* LpxT has dual positional specificity modifying lipid A at positions 1 and 4' [120]. Also, LpxT activity in *P. aeruginosa* is modulated by the addition of L-Ara4N residues to lipid A mediated by the transferase ArnT, which is upregulated under magnesium limitation.

The lipid A phosphate at the 1-position can be also methylated, such as in *Leptospira interrogans* (Fig. 3) [121]. LmtA, the protein that catalyses this modification, uses an S-adenosylmethionine donor. However, unlike the LpxT phosphorylation, LmtA-mediated methylation occurs on the cytoplasm prior to the transport of lipid A core across the inner membrane.

MODIFICATION OF KDO_2-LIPID A ACYL CHAINS

Changes in the number of acyl chain of the lipid A molecule, by removal or addition, represent another way to remodel the Kdo_2-lipid A molecule (Fig. 3). There are three enzymes that are involved in modulating the number of acyl chains in the lipid A. PagP is responsible for an increase in acyl chain numbers [122, 123], whereas PagL and LpxR catalyse the removal of acyl chains [124, 125]. These proteins are in the outer membrane, and they are part of a superfamily of β-barrel enzymes with established structure-function relationships suggesting a general role in adaptive responses to outer membrane stress [126].

PagP is a phospholipid::lipid A palmitoyltransferase that adds a palmitoyl residue (C16:0) from the *sn*-1 position of a phospholipid to the hydroxyl group on the R-3-hydroxymyristate chain at position 2 of lipid A (Fig. 2b). This reaction is under the regulation of PhoPQ [122]. PagP is an eight-stranded β-barrel with long extracellular loops and a short N-terminal amphipathic α-helix [127, 128], which sits in the membrane with the barrel axis tilted at 25° [126]. The core of PagP contains a hydrocarbon ruler that resides in the interior of β-barrel towards the LPS exposed region of the protein and provides remarkable substrate selectivity [129]. PagP homologues are found in several Gram-negative bacteria [126, 130]. PagP activity in *Salmonella* PagP has been associated to resistance to antimicrobial peptides [122] and also reduces Toll-like receptor 4 activation [131]. PagP also contributes to evasion from the innate immune system in *Legionella pneumophila* [132] and *B. bronchiseptica* [133] infections. The palmitoylation of *P. aeruginosa* lipid A is commonly found in cystic fibrosis isolates, suggesting it plays a critical role for bacterial adaptation to chronic lung infection [134]. The *P. aeruginosa* PagP transfers a palmitate to the hydroxy acyl chain at the 3′-position (Fig. 2b) [135]. This protein also illustrates the divergence of PagP since while it does not share amino acid sequence identity with other known PagP enzymes, the β-barrel tertiary structure with an interior hydrocarbon ruler is conserved [135]. Further, the location of the palmitate in the 3′ position of lipid A, may promote an elevated pro-inflammatory response based on increased cytokine production, which is not observed with other palmitoylated lipid A species.

In *Bordetella* species, PagP is regulated by Bvg two-component system and the PagP proteins in members of this genus can have strict or dual specificity for the attachment site in the lipid A. *B. bronchiseptica* PagP adds C16:0 at the 3′-position whereas *Bordetella parapertussis* PagP transfers palmitates to the lipid A C-2 and C-3′ positions [136]. The *B. bronchiseptica* PagP modification is required for resistance to antibody-dependent complement-mediated killing in a murine model of infection. The dual specificity of PagP in *B. parapertussis* contributes to the heterogeneity in lipid A structures observed in this bacterium, with penta- and hexa-acylated structures containing one and two palmitates, respectively. These modifications also influence antimicrobial peptide resistance and LPS endotoxicity via TLR4 receptor signalling.

PagL is an outer membrane enzyme that removes the 3-*O*-linked acyl chain from lipid A (Fig. 2b). The activity was initially discovered in *R. leguminosarum* [137], but the gene was first identified in *S. enterica* using a PhoP constitutive mutant [125]. Like PagP, PagL activity decreases the endotoxic activity of in *Salmonella* LPS [131]. Also, loss of PagL function in *P. aeruginosa* has been observed during chronic adaptation to the cystic fibrosis airways [138]. However, the *Salmonella* PagL does not function *in vivo* since PagL activity is strongly inhibited by the presence of L-Ara4N in lipid A [139]. This inhibition is associated to interactions between PagL extracellular loops and L-Ara4N-modified lipid A [140]. The *P. aeruginosa* PagL crystal structure has been solved and reveals a fold like PagP [141]. However, PagL has a different way to present its lipid substrate to the catalytic site on the β-barrel exterior [141], and unlike PagP, PagL does not have a strict acyl chain preference [142].

LpxR is also a β-barrel outer membrane protein that removes the 3′-acyloxyacyl unit in lipid A (Fig. 2b); it was identified in *Salmonella* and other enteric pathogens [124]. The enzyme requires Ca^{2+} as a cofactor and its activity can only be detected under when the bacteria reach stationary phase, suggesting growth phase dependent regulation [124]. When macrophages were infected with stationary phase bacteria, the intracellular growth of the *lpxR*-null strain was lower than that of the wild-type strain, and also associated with higher expression level of inducible nitric oxide synthase, suggesting that lipid A 3′-*O*-deacylation is beneficial for intracellular growth [143]. Similarly, LpxR is important for motility and invasion of *Yersinia enterocolitica* [144]. As with PagL, modelling experiments suggest that the active site of LpxR cannot accommodate L-Ara4N-modified Kdo_2-lipid A [144]. The crystal structure of the *S. enterica* LpxR shows a 12-stranded β-barrel with the active site located between the barrel wall and a α-helix from an extracellular loop [145]. Modelling the substrate on the active site coupled to site-directed mutagenesis supports the notion that LpxR has a catalytic mechanism similar to that of phospholipase A2 [145], which is another important outer membrane β-barrel enzyme involved in lipid bilayer homeostasis [126].

Another acyl chain modification involves the addition of a hydroxyl group to the 2-position of the 3′-linked secondary acyl chain (Fig. 2b). Early structural studies in many bacteria have documented the presence of hydroxylated secondary linked acyl chains in their lipid A [146–149]. This modification depends on the LpxO, an enzyme that catalyses hydroxylation in an oxygen dependent manner at the cytoplasmic surface of the inner membrane [150]. LpxO is a member of the Fe^{2+}/O^2/α-ketoglutarate-dependent dioxygenase family [151] originally identified in *S. enterica*, but with homologues present in many bacterial species. In *B. cenocepacia*, LpxO homologues are involved in the synthesis of 2-hydroxylated derivatives of phosphatidylethanolamine and ornithine-containing lipids, but do not appear to modify lipid A [152], suggesting that LpxO proteins may have several functions in membrane lipid synthesis. In *Klebsiella pneumoniae* and *A. baumannii*, lipid A hydroxylation increases resistance to cationic antimicrobial peptides, such as polymyxins, and is required for full virulence, as demonstrated in the insect *Galleria mellonella* infection model [153, 154]. Similarly, two LpxO homologues in *P. aeruginosa*, are required for virulence in *G. mellonella* [155]. Lipid A hydroxylation is also important for *Burkholderia pseudomallei* replication within macrophages [156], for *K. pneumoniae* persistence in the lung of experimentally infected mice [157] and to modulate *V. cholerae* innate immune recognition [158]. Clinical *Enterobacter cloacae* complex isolates, especially those displaying inherent colistin resistance show lipid A hydroxylation when grown in medium without the antimicrobial peptide, suggesting that this lipid A modification is constitutively expressed in these strains [159]. Remarkably, the presence of an R-2-hydroxymyristic acid moiety on lipid A structure of bacteremic *E. cloacae* complex isolates was directly associated with mortality in neonates with septic shock, linking lipid A structure structures with sepsis outcome [160].

How lipid A hydroxylation contributes to infection remains an open question. The effect of lipid A R-2-hydroxymyristate modification could be to increase hydrogen bonds between adjacent lipid A chains, which would increase the membrane's resistance to the penetration of antimicrobial cationic peptides. It has been proposed that during infection, R-2-hydroxymyristic acids released by LPS through leucocyte acyloxyacyl hydrolase would be converted to 2-hydroxymyristoyl coenzyme A, a potent inhibitor of *N*-myristoyl transferase that is required for cell signalling, which would potentially lead to cell death [150]. However, several observations suggest the importance of LpxO-dependent modification in immune evasion. In *A. baumannii*, 2-hydroxylation of lipid A limits the activation of the mitogen-activated protein kinase Jun N-terminal protein kinase and mediates the production of the anti-inflammatory cytokine interleukin-10 (IL-10), resulting in a reduction of inflammatory responses upon infection [153], while lack of lipid A hydroxylation in a *Burkholderia multivorans* isolate was associated to reduced cytokine activation [161].

KDO_2 MODIFICATIONS

The number of Kdo residues attached to the lipid A molecule can vary depending on the functionality of WaaA, as described above, but also by the removal of Kdo residues. Kdo hydrolases have been demonstrated in *H. pylori* [162, 163] and *Francisella* [164]. Removal of the outer Kdo residue occurs at the periplasmic side of the inner membrane [163]. The biological role of the removal of Kdo residues is unclear. However, inactivation of the *H. pylori* Kdo hydrolase activity affected antimicrobial peptide resistance and the expression of surface Lewis X and Y epitopes by a mechanism not well understood [163].

E. coli and *S. enterica* modify their outer Kdo sugar by transferring a PEtn residue (Fig. 3). This reaction occurs in response to high Ca^{2+} concentrations in the growth medium and is catalysed by EptB, which has high sequence homology to EptA [165]. Inner core Kdo and heptoses can also be modified by PEtn residues, which are incorporated directly to the sugars or the substituting phosphates. Examples of these modifications are found *N. meningitidis* and *Neisseria gonorrhoeae*, *H. influenzae*, and a wide range of Gram-negative bacteria [166–168]. The corresponding enzymes share a high degree of similarity.

Like lipid A, the inner core LPS can also be modified by the addition of negatively charged molecules. Certain bacteria, such as *H. influenzae*, *V. cholerae* and *Pasteurella multocida*, synthesise LPS with a single Kdo residue in the inner-core region, which is phosphorylated at position 4 by a Kdo kinase (KdkA) [64, 65, 71, 169–171] that is evolutionary related to eukaryotic protein kinases, similarly to the *E. coli* and *S. enterica* WaaP [171, 172]. WaaP transfers a phosphate to position 4 of heptose I and this reaction is a prerequisite for the sequential addition of heptose III to the inner-core oligosaccharide by WaaQ, and another phosphate residue to position 4 of heptose II by WaaY [173, 174]. *P. aeruginosa* synthetises a highly phosphorylated inner core with

three phosphate groups at positions 2 and 4 of heptose I, and position 6 of heptose II [175, 176]. The phosphorylation at position 4 of heptose I is also catalysed by a WaaP homolog [177, 178], and is critical for bacterial viability [178] and the export of LPS to the outer membrane [179]. Therefore, substitutions in the inner core by phosphate residues are molecular checkpoints for the subsequent assembly of a complete core LPS, and they play a role in outer membrane stability and virulence [71, 171, 178, 180, 181].

In *Rhizobium*, RgtA and RgtB are responsible for adding two galacturonic acid molecules to the outer Kdo sugar [182, 183]. RgtA and RgtB use dodecaprenyl-phosphate-galacturonic acid as a donor [183]. Additionally, other bacteria modify the inner core sugars by the addition of galacturonic acid, which becomes an alternative way to provide negative charges required for outer membrane stability [8]. In contrast, Kdo or Ko can be modified by the addition of L-Ara4N (Fig. 2). Although, it is assumed these reactions are catalysed by ArnT homologues [83], this has not been directly demonstrated.

CONCLUDING REMARKS

In summary, the Kdo_2-lipid A molecule, and additional proximal residues of the LPS core oligosaccharide display a wide range of heterogeneity reflecting bacterial adaptation to environmental conditions including survival in the host during pathogenic or symbiotic interactions. For example, recent evidence shows that the cumulative effects of LPS in the microbiota of intestinal crypts has a beneficial modulatory effect in proliferation vs differentiation balance of intestinal epithelial cells specific microbiota [184]. Therefore, developing molecular approaches to investigate the Kdo_2-lipid A chemical '*modifysome*' will help us better understand the dynamics of polymicrobial host-bacteria interactions in health and disease states.

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Conflicts of interest
The author declares that there are no conflicts of interest.

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