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Bacterial Carbohydrate Diversity – A Brave New World

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Abstract

Glycans and glycoconjugates feature on the “front line” of bacterial cells, playing critical roles in the mechanical and chemical stability of the microorganisms, and orchestrating interactions with the environment and all other living organisms. To negotiate such central tasks, bacterial glycomes incorporate a dizzying array of carbohydrate building blocks and non-carbohydrate modifications, which create opportunities for infinite structural variation. This review highlights some of the challenges and opportunities for the chemical biology community in the field of bacterial glycobiology.

Keywords

bacterial glycome; carbohydrate database; metabolic engineering; glycan array; heptose-1,7-bisphosphate; antibiotic resistance

Introduction

The human glycome is frequently deemed to be a labyrinthine nightmare. Those glycans are based on 10 unique monosaccharide building blocks, which can be combined into oligomers and polymers with linear and branched structures joined by glycosidic linkages between the various hydroxyl groups, with each glycosidic linkage adopting either α- or β-anomeric configuration. The structures may additionally be subject to modifications including, for example, sulfation and phosphorylation [1,2]. Despite this complexity however, when it comes to comparisons of carbohydrate diversity between prokaryotic and eukaryotic life forms, prokaryotes win hands-down. There are hundreds of unique monosaccharide building blocks and chemically-defined saccharide modifications in the database of naturally-occurring carbohydrates (http://csdb.glycoscience.ru/bacterial/) [3,4]; the vast majority of these are prokaryote-specific. The carbohydrates that feature in bacterial glycans range from trioses to dodecoses (Figure 1a) and amongst these, the hexoses show extensive variation in

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Conflict of interest

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substitution patterns and the heptoses and octoses are exclusively prokaryotic. The nonulosonic acids, which are derived biosynthetically from the hexoses, are also far more varied: there is a single example in man, but dozens have been characterized to date in various bacteria [5]. In addition, a plethora of modifications including alkyl, acyl, amino acyl, phosphoryl and even nucleoside groups are often found decorating the carbohydrates [4]. Defining and cataloging this variety, not to mention understanding its significance, is a Herculean task.

Bacterial carbohydrates may be components of repeating glycopolymers or, more complex glycoconjugates, which may reveal elaborate “samplers” of different sugars (Figure 1b) [6]. In cells, the glycans commonly include cell-surface structures that are essential for the mechanical integrity of bacterial cells and for critical interactions with other bacteria and the hosts with which they coexist [7,8]. The key glycoconjugates in Gram-negative bacteria are peptidoglycan (PG), lipopolysaccharide (LPS), lipooligosaccharide (LOS), capsular extracellular polysaccharide (EPS), capsular polysaccharide (CPS), and N- and O-linked glycoproteins. In contrast, Gram-positive organisms lack LPS and LOS but instead feature glycosylated lipo- and wall techoic acids (LTAs and WTAs), which are anionic copolymers of glycerol or ribitol phosphate and carbohydrates linked via phosphodiester linkages, conjugated to either PG (for WTA) or diacyl glycerol (LTA). The Gram-positive mycobacteria show additional glycan variations including arabinogalactan and lipoarabinomannan conjugates, which feature many furanose sugars [9].

This opinion summarizes recent examples of the importance of chemical biology approaches for identifying the fates of bacterial sugars in pathogen glycoconjugates, investigating carbohydrate-based interactions, defining the activity of bacterial monosaccharides in eukaryotic signaling, and highlighting the significance of glycan modifications in antibiotic resistance.

Enlisting metabolic labeling for tracking and analyzing bacterial cell-surface glycomes

Metabolic oligosaccharide engineering (MOE), involves delivery of cell-permeable biosynthetic precursors of glycan constituents into living cells and organisms [10]. MOE reagents are commonly modified with azides or terminal alkynes, which serve as biorthogonal reactivity handles for the introduction of epitope, biotin, or fluorescent tags. MOE has proven extremely valuable in research on the glycobiology of mammalian cells and model organisms. For example, studies with peracetylated derivatives of C-2 acetamido hexoses, in which the *N*-acetyl groups are replaced with *N*-azidoacetyl are useful for labeling eukaryotic *N*- and *O*-linked glycoproteins [10]. For these efforts, knowledge of cellular pathways for conversion of carbohydrate analogs into nucleotide-activated sugars for glycoconjugate assembly is crucial. Notably, the in-cell conversion of free *N*-acetyl mannosamine into CMP-*N*-acetylneuraminic acid (CMP-NeuNAc), a non-2-ulosonic acid (NulO) [11,12] has been extensively exploited as it is commonly associated with mammalian cell-surface glycoproteins. NeuNAc is the only nine-carbon sugar in man with the main variation in non-human mammals being the *N*-glycolyl derivative [13]. MOE in mammalian

cells is also enabled by a salvage pathway that affords phosphorylation of unmodified hexoses to generate precursors for conversion into UDP-sugars [14]. This pathway may not be broadly present bacteria and so cannot always be relied upon for MOE.

Information on the pathways for glycoconjugate biosynthesis in bacteria has led to important avenues for selective MOE in bacterial pathogens. Prokaryote-specific NulOs, feature prominently in cell surface-glycoconjugates. NulO biosynthesis follows a common logic across domains of life, with the key step involving a three-carbon extension of a six-carbon nucleotide sugar with phosphoenol pyruvate (PEP). For example, CMP-pseudaminic acid (CMP-Pse) [15] and CMP-legionaminic acid (CMP-Leg) [16] are biosynthesized from UDP-GlcNAc and GDP-GlcNAc, respectively, via the intermediacy of tri-deoxy-diacetamido-hexoses (Figure 2a). Biochemical manipulation of bacterial hexoses, such as those in the Pse and Leg pathways, commonly involve site-selective oxidation of hydroxyl groups (at C3 or C4), which in turn enables further biochemical manipulation such as elimination, enolization and reprotonation for epimerization or ketoisomerization, and transamination [17].

In bacteria, NulOs play important roles in virulence and pathogenicity and may function in carbohydrate mimicry foiling the human immune system [5]. Bacterial NulOs are common modifications of the capsular polysaccharide (CPS) and O-antigen, and are attached to the Fla proteins in flagellar appendages that are essential for motility. Critical to successful MOE is the provision of cell-permeable precursors, commonly as neutral, per-O/N-acetylated derivatives of hexose or hexosamine intermediates, which are de-O-acetylated by cellular esterases and enter into endogenous pathways for conversion into nucleotide-activated sugars for glycan assembly. Recent MOE studies targeted at the Pse pathway, employing 2- and 4-azidoacetyl analogs of the 2,4-diNAc-L-altrose (2,4-diNAcAlt) intermediate [18], showed superior incorporation of 2-NAc-4NAz-Alt (**1**, Figure 2b) into LPS and glycoproteins by MOE. Strain-specific metabolic labeling and fluorescence-based imaging of the PA1244 strain of *Pseudomonas aeruginosa* was also realized. Additionally, earlier MOE on the Leg pathway showed that C-6 azide modification of the neutral 6-deoxyhexose intermediate *en route* to legionaminic acid (**2**, Figure 2b), could also be applied for strain-selective labeling of strains of *Legionella pneumophilia* [19].

MOE has also been applied to 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), which is found in the inner core of the LPS of Gram-negative bacteria. Kdo is biosynthesized through a PEP-based extension of a pentose sugar (Figure 2c) and incorporation of the 8-azido analog of Kdo (Kdo-8-N_3) (**3**, Figure 2a) into the *E. coli* LPS has been validated [20]. Recognizing that Kdo is a specialized carbohydrate in Gram-negative bacteria, Chen and coworkers employed MOE with Kdo-8-N_3 to distinguish between Gram-negative and Gram-positive bacteria in the mouse gut microbiota [21]. In these studies, Gram-negative bacteria were labeled *in vivo* by MOE with Kdo-8-N_3, followed treatment of fixed tissue with an alkyne-TAMRA reagent for click chemistry (Figure 2d). Gram-positive bacteria were visualized with a fluorescent derivative of vancomycin (Vanco-Bodipy), which binds more readily to the surface-exposed peptidoglycan relative to the periplasmic peptidoglycan of Gram-negative bacteria. Recently, it has been reported that transport of Kdo-8-N_3 into *E. coli* is mediated by the sialic acid transporter NanT and that uptake of Kdo-8-N_3 into pathogens, including *P. aeruginosa* and *Acinetobacter baumannii*, which lack NanT, can be improved by

heterologous expression of *E. coli* NanT [22]. This genetic manipulation will expand the scope of MOE with Kdo for the study of Gram-negative pathogens.

In summary, the growing understanding bacterial carbohydrate diversity across bacterial phyla suggests that MOE holds considerable promise for the species and strain-selective labeling of bacteria that reaches far beyond the simple crystal violet Gram-stain introduced at the end of the 19th century.

Cellular signaling by bacterial monosaccharides

Recently, there have been intriguing observations on the pathogen-associated molecular pattern (PAMP) activity of soluble bacterial carbohydrates in eukaryotic cells. Host recognition of PAMPs is important for initiating innate immune responses that are critical for eliminating pathogens and for the deployment of adaptive immunity processes [23]. PAMPs are commonly associated with Gram-negative LPS and Gram-positive LTA glycoconjugates.

In 2015 it was reported that mammalian cells detect and respond to a heat-resistant factor behaving as a PAMPs [24]. This factor was determined to be heptose-1,7-bisphosphate (HBP). HBP is an intermediate in the biosynthesis of an ADP-heptose, which is a key precursor in the LPS pathway [25] (Figure 3a). Studies in various pathogens revealed that HBP can be delivered into host cells by different mechanisms including *via* the *cag* Type 4 secretion system (*cag* TSS4) of *Helicobacter pylori* [24,26], by transfer across membranes from *Neisseria* [24], and by direct secretion from the intracellular pathogen *Shigella flexneri* [27]. Regardless of the mode of delivery, HBP is a marker of bacterial activity and triggers a signaling cascade, which involves alpha-kinase 1 (ALPK1) followed by the phosphorylation-dependent oligomerization of the tumor necrosis factor (TNF-α) receptor–associated factor (TRAF)–interacting protein with the forkhead-associated domain (TIFA). These processes ultimately activate proinflammatory responses via transcriptional activation by NFκB. As the response is exclusive to Gram-negative pathogens with an ADP-heptose pathway, its presence is proposed as representing a means of differentiating between Gram-negative pathogens and the beneficial microbiota (Figure 3b).

Bacterial glycan arrays

Glycan arrays are a mainstay of glycobiology research and the capabilities of this technology are being continually evolved to meet demands [28]. The arrays provide a highly efficient means of screening carbohydrate-binding proteins (GBPs) to identify their cognate carbohydrate ligands. Arrays of GBPs are also valuable and can be enlisted for the identification of glycans serving as signals in physiological interactions and responses. Glycan arrays were initially focused on mammalian glycans in recognition of the involvement of cell-surface carbohydrates in human disease [28]. However, the importance of *bacterial* cell-surface glycans in infection and inflammation has led to increased interest in the development and application of bacterial glycan arrays [29]. Bacterial glycan arrays may be specifically targeted to a particular genus or a species. For example, a recently-introduced array of synthetic glycans representing mycobacteria has been developed and

employed to investigate interactions between the human innate and adaptive immune systems and diverse components of the glycome of *Mycobacterium tuberculosis* [30]. Bacterial glycan arrays, have also been developed from fractionated, but uncharacterized glycoconjugates; these are known as “shot-gun arrays” [29,31]. Although uncharacterized, these types of arrays may provide insight into carbohydrate that might be of interest in a particular type of glycoconjugate.

A new glycan array featuring linear and branched glycans (1-20 carbohydrates in size), designated as the Max Planck Society (MPS) array, has also been introduced [32]. The collection of 300 glycans includes those derived from automated and solution phase synthesis, chemoenzymatic synthesis and bacterial isolates. From this set, 140 glycans representing mammalian, protozoal and prokaryote-specific glycans has been validated in array format using plant lectins and applied to glycoimmunology and infectious disease glycobiological screens. The glycans and the metrics for the diversity of the MPS array are noted to be comparable to those from other centers. Future steps, towards the assembly of glycan arrays that represent the rich diversity of bacterial glycans will certainly contribute to a broader understanding of the implications of this diversity in human disease.

In this context, bioinformatics analyses [33], which exploit the current comprehensive glycan database of Toukach [4], and codify the most common, but unique bacterial glycans, will guide *de novo* and chemoenzymatic synthesis efforts to build the most relevant arrays. From these analyses it is evident that although entire prokaryotic glycomes are too extensive to screen with currently-available technologies, when arrays focus on smaller motifs such as disaccharides, the structure-space becomes much more tractable, while maintaining diversity. Other recent advances in glycan presentation also address challenges with bacterial glycan screening technologies. For example, a recent multiplex mammalian glycan bead array (MGBA) system, which is based on Luminex bead array technology, might be advantageously pivoted towards bacterial glycans [34].

Antibiotic resistance through strategic glycosylation and glycan modification

The forms and functions of bacterial glycans are even more expanded by carbohydrate modifications that occur either on saccharide building blocks prior to glycan assembly, or, by modification of assembled glycans. (See Figure 1b for a limited set of modifications.) As glycoconjugates represent the “front line” for interactions with the environment, other organisms and natural and synthetic antibiotics, these additional avenues for structural variation enable the evolution of new structures that are adapted and selected for survival under challenging conditions.

The LPS of Gram-negative bacteria contributes to structural integrity and protects the membrane from access by many small molecule antibacterial agents. The LPS features many negatively-charged functional groups including phosphoryl groups on the lipid A and heptose sugars and carboxyl groups on the Kdo sugars (Fig 1b). Alteration of this charge density, through glycan modification, affords resistance to antibiotics and antimicrobial peptides. A deceptively-simple modification, that serves to modulate glycoconjugate charge,

is installation of the phosphoethanolamine (PEtN) moiety found as a site-specific modification on carbohydrates including hexoses, heptoses and Kdo in diverse glycoconjugates. Several PEtN transferases have been characterized to date. Recently two PEtN transferases representing a new family have been identified by bioinformatics analysis and characterized from *Pasteurella multocida* [35]. These enzymes transfer a single PEtN to Kdo in LPS and are associated with resistance to the cationic antimicrobial peptide cathelicidin-2.

Alternatively, the core peracylated β-1,6-glucosamine disaccharide diphosphate phosphate of the LPS may be modified to introduce positively-charged moieties. This is illustrated with the action of ArnT, a periplasmic glycosyl transferase that confers resistance to the cationic antibiotic polymyxin in *Escherichia coli* and *Salmonella enterica*, as well as in serious pathogens including *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [36]. Resistance results from transfer of the cationic pentose sugar, 4-amino-4-deoxy-L-α-arabinose (Ara4N, PEN*f*, Figure 1b) from undecaprenol phosphate-α-L-Ara4N (UndP-Ara4N) to phosphoryl groups in the assembled $(Kdo)_2$-peracylated Lipid A. This modification alters the net negative charge on the LPS and thus susceptibility to the highly-basic antibiotic. The structure of the integral membrane ArnT has recently been characterized providing mechanistic insight and details of the binding of the UndP-Ara4N and the peracylated lipid A derivative [37]. Ara4N is an unusual pentose (in pyranose form) that is biosynthesized from UDP-glucuronic acid by C4 oxidation, which facilitates C-6 decarboxylation. Then pyridoxamine-dependent transamination affords UDP-α-L-Ara4N, which is converted to UndP-Ara4N [38]. This process illustrates how the pyranose-form pentose sugars are biosynthesized from a common UDP-sugar without recourse to the metabolic pool of hexoses.

The biosynthesis of another unusual pentose, methylthio-D-xylose (MTX), has also been recently elucidated [39]. MTX originates from 5'methyl thioadenosine, a byproduct of mammalian polyamine biosynthesis. MTX is found as an α-1-4-linked modification of terminal mannoses on the lipoarabinomannan (LAM) polysaccharide in the cell envelope of *Mycobacterium tuberculosis* and other pathogenic mycobacteria. The uniqueness of the MTX motif has been targeted in the development of a novel immunoassay for *M. tuberculosis*. The assay is selective for *Mycobacterium tuberculosis* and shows no cross-reactivity with fast-growing mycobacteria or other bacteria [40]. The cell-surface presentation of the MTX-modified cap suggests that it plays a role in host–pathogen interactions and, although the current understanding of the function of MTX is still limited, knowledge of the biosynthetic pathway and the development of MTX-deficient *M. tuberculosis* strains will be critical for future studies to understand role physiological role of this rare pentose.

Conclusions

The cell-surface glycans and glycoconjugates of bacteria play essential roles in the mechanical and chemical stability of microorganisms and are intimately involved in interactions with the environment and other living organisms. In particular, the glycomes of pathogens and symbionts are of great interest as they provide opportunities for infinite

structural variation to adapt to the hosts that they inhabit and thus the details of these variations are extremely important to human health.

Major progress has been made in determining the structures of complex carbohydrates through advances in biochemical, analytical and bioinformatics approaches. Despite this, current surveys suggest that the known diversity of bacterial carbohydrates, carbohydrate modifications and the glycans into which they are embedded may be just the “tip of the iceberg” and, there is the anticipation that an understanding of the sheer scope of bacterial glycomes will follow apace with advances in bacterial genome sequencing where the metrics are well-defined [41]. In addition to providing new information on glycan diversity, the surge of genomic and metagenomic data will fuel prediction of additional carbohydrate-related pathways that contribute to the biosynthesis of bacterial glycans, as genes for the biosynthesis of unique bacterial sugars are commonly clustered with those that program glycoconjugate assembly.

In the face of this progress there remain major challenges to understanding the myriad functions of carbohydrates in bacteria. As highlighted in this opinion, chemical biology approaches, including glycan arrays and metabolic oligosaccharide engineering, show great promise for defining bacterial glycomes and their interactions. In addition, the recent discovery of bacterial carbohydrates as mammalian signaling molecules and the specific roles of glycan modifications in antibiotic resistance are illustrated and suggest that there are many surprises yet to come in the Brave New World of bacterial carbohydrates.

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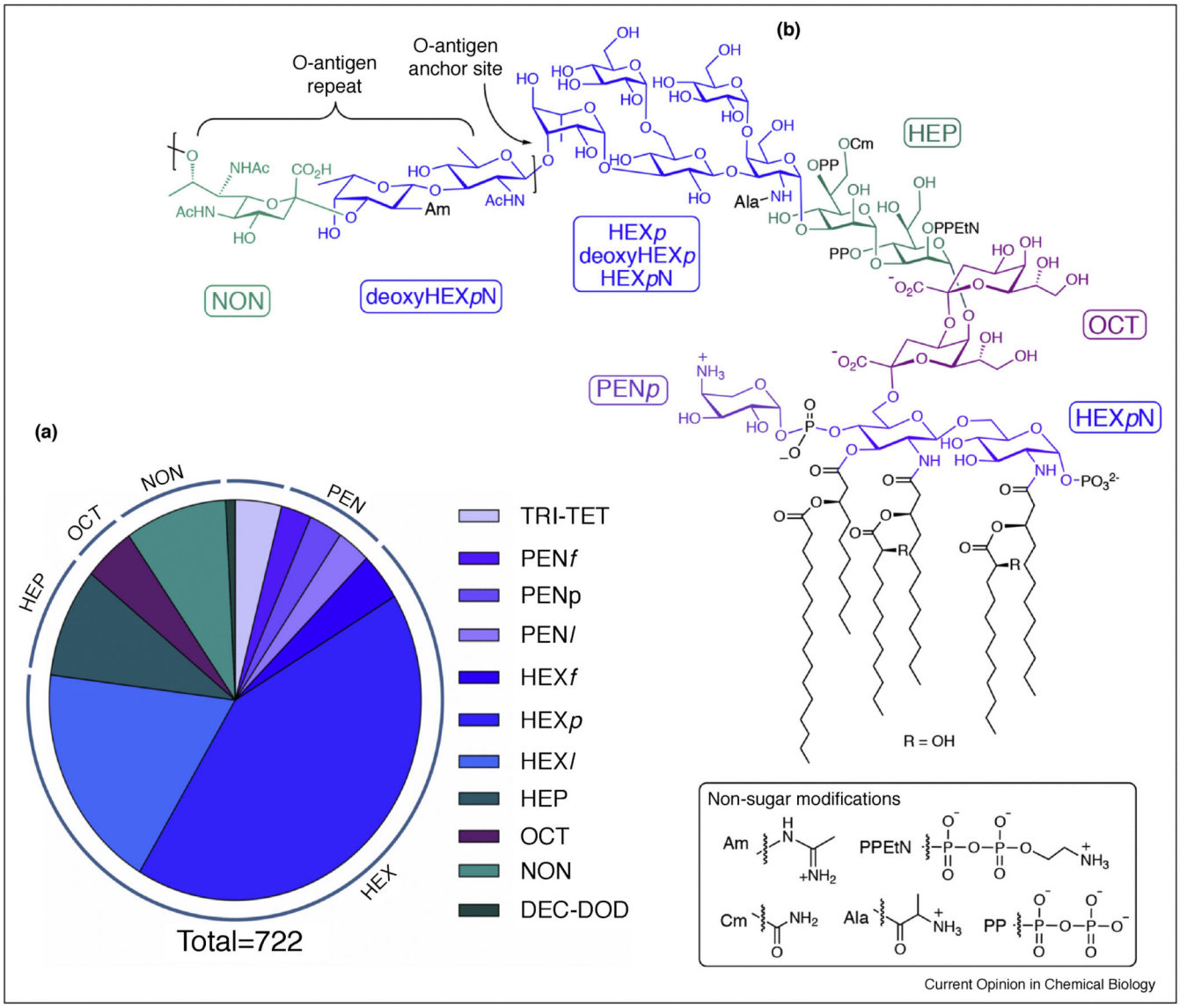

Figure 1.

Bacterial carbohydrate diversity.

(a) Bacterial carbohydrate distribution based on size. The current BCSDB (http://csdb.glycoscience.ru/bacterial/) includes over 700 unique carbohydrates ranging from trioses to dodecanoses [4]. Pentoses and hexoses are further differentiated as furanose (*f*), pyranose (*p*) and acyclic/unknown (*l*). (b) The structure of a lipopolysaccharide (LPS smooth) from *Pseudomonas aeruginosa* (O12 serotype) exemplifies the diversity of carbohydrates in a single glycoconjugate [6]. *O*-Acetyl groups are not shown. An Arap4NP is shown on the Lipid A diglucosamine core for the purpose of illustrating the modification. This carbohydrate is known to be associated with *P. aeruginosa*, but not specifically documented in the serotype illustrated. Further glycan variation is achieved by carbohydrate modifications for example with amidino (Am), carbamoyl (Ca), phosphatidyl ethanolamine (PEtN), diphosphate (PP) and alanyl (Ala).

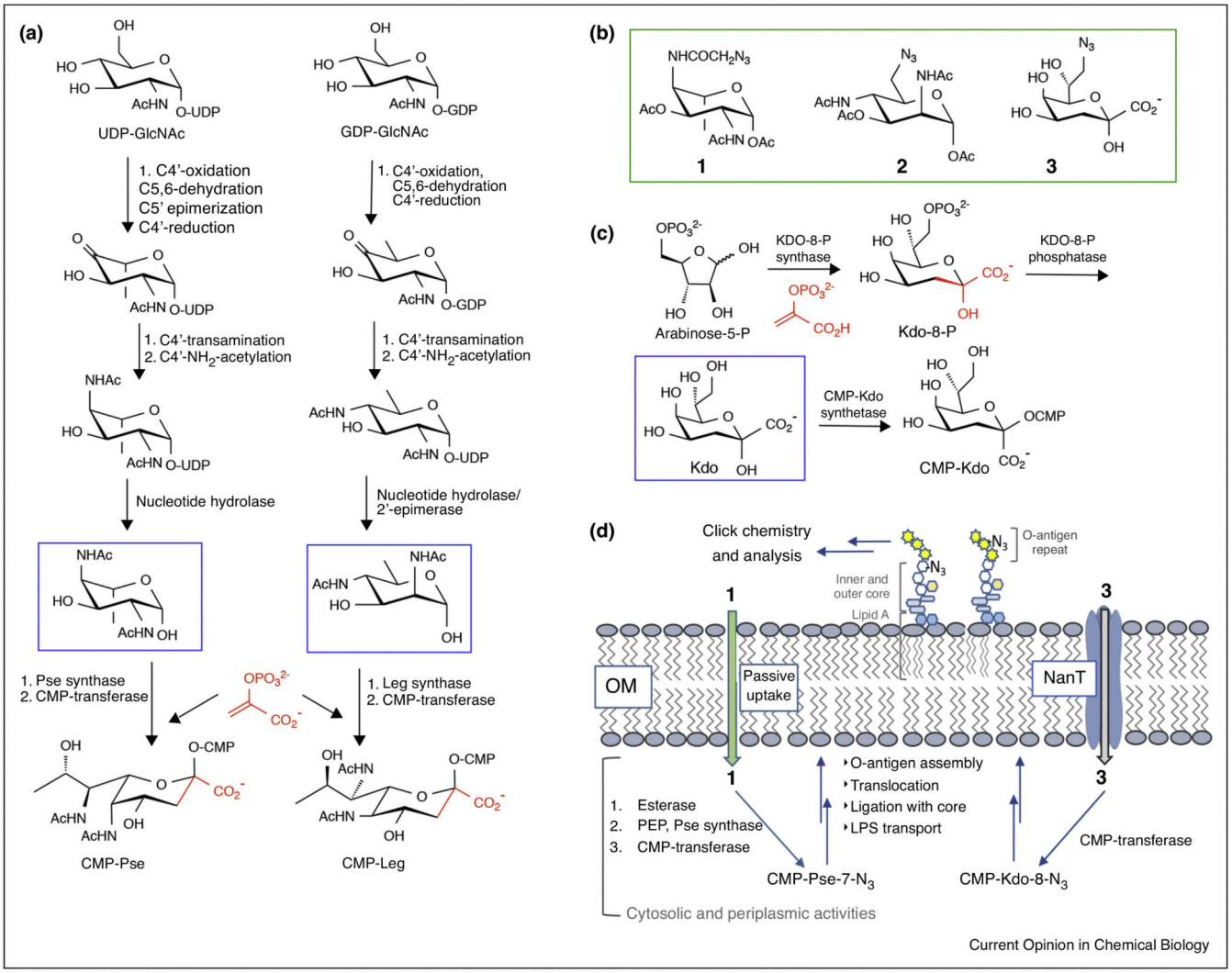

Figure 2.

Knowledge of carbohydrate biosynthetic pathways in bacteria provides important information on precursors for metabolic oligosaccharide engineering (MOE).

(a) Pseudaminic and legionaminic acid biosynthesis via three-carbon extension (PEP – shown in red) of hexose precursors *via* the Pse (*Helicobacter pylori*) and Leg (*Campylobacter jejuni*) pathways. (b) Azide-labeled MOE precursors **1**, **2**, and **3** for Pse, Leg and Kdo pathways (green box). (c) Octulosonic acid biosynthesis (*E. coli)* via a three-carbon extension (PEP – shown in red) of a pentose precursor. (d) Cellular uptake and fate of **1** and **3** in bacterial MOE studies.

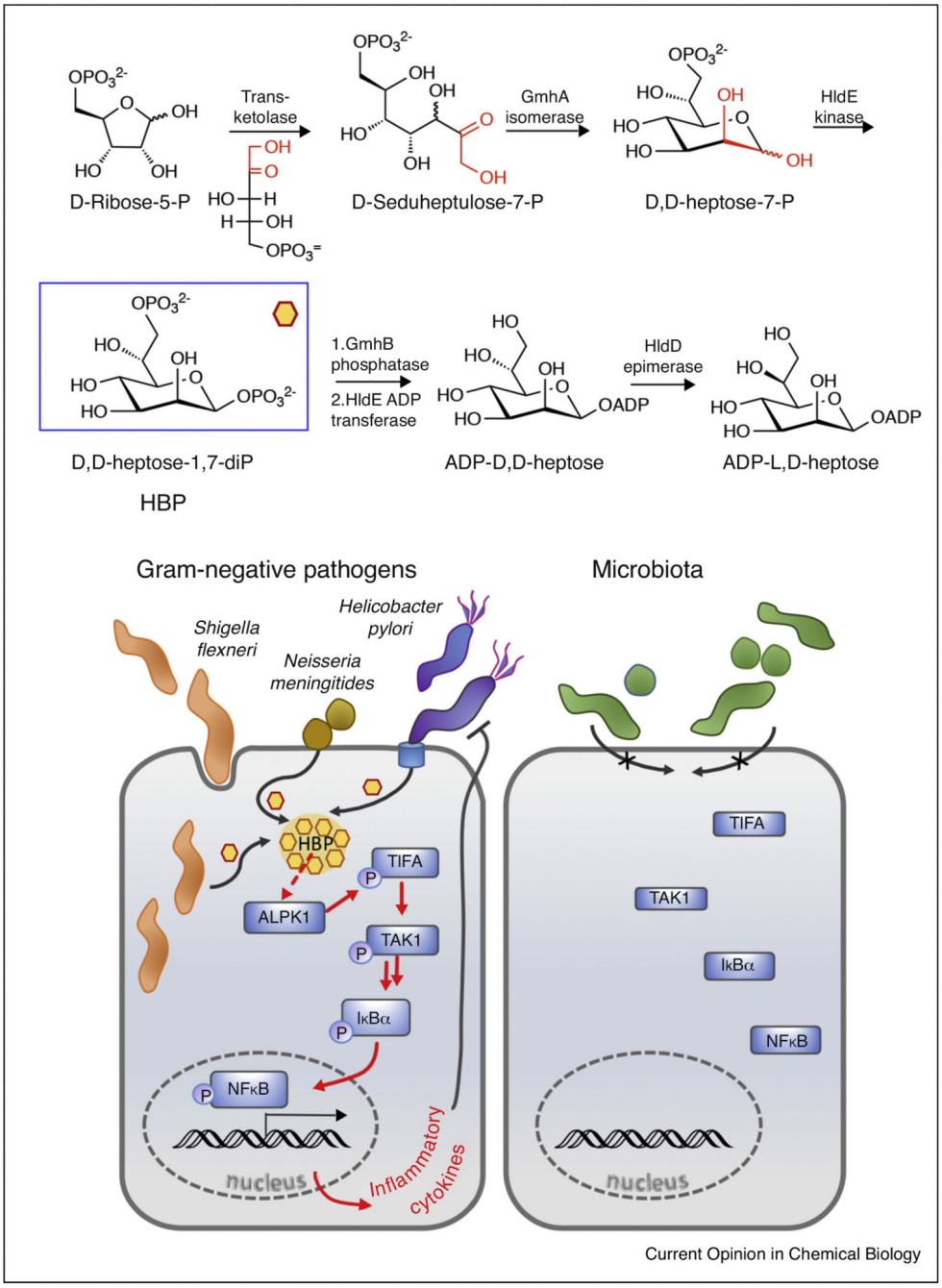

Figure 3.

Cellular signaling with a bacterial monosaccharide.

(a) The ADP-heptose biosynthesis pathway illustrating the transketolase step, which establishes the seven-carbon skeleton. The key intermediate, HBP, is highlighted in a blue box. (b) Cellular delivery of bacterial HBP by various pathogens and signaling cascade initiated by activation of ALPK1 ultimately leading to transcriptional regulation via NF-κB stimulating an inflammatory response.