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Predicting the Biological Repeating Unit of Bacterial Lipopolysaccharides: Common Structural Motifs in Regular Polysaccharides from Different Genera

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Abstract

The cell surface of most organisms is covered by carbohydrates linked to lipids or proteins. In gram-negative bacteria there are generally two kinds of carbohydrate molecules covering the cell surface; lipopolysaccharides (or occasionally lipooligosaccharides) and capsular polysaccharides. Whilst the capsular polysaccharides, when present, are very long and loosely attached to the cell surface the lipopolysaccharides are anchored in the cell membrane and protrude perpendicular to it. Thus, in absence of a capsule, the surface presented to other microbes and the immune system is dominated by the ends of the lipopolysaccharide molecules. Here we show that structures presented to the environment are shared among a wide range of different bacteria and can often be predicted from the chemical structure of the polymer.

Introduction

The importance of lipopolysaccharides (LPS) for the survival of bacteria in their natural surrounding has long been recognised. There is however no consensus regarding their function *in vivo* and presumably they have several roles such as protection against bacteriophages, surface adhesion and resistance towards a variety of other environmental stresses [1]. Lipopolysaccharides are some of the most variable molecules produced by bacteria. The number of components in bacterial polysaccharides is staggering; a 1990 review counts 85

different glycosyl residues (excluding substituents) [2], which leads to an enormous number of possible structures. Despite this the diversity identical or similar structures are encountered more often than would be expected. Even when the limitations imposed by biosynthesis are taken into account there are structural themes that are more common and wide spread than might be anticipated. In the case of *E. coli* approximately 15% of the known O-antigen structures are identical to structures from other genera and common fragments causing serological cross reactivity are relatively abundant [3].

The Biological Repeating Unit

Lipopolysaccharides can be divided roughly into three parts; lipid A (endotoxin) – the part that attaches the LPS to the membrane, the O-antigen that consists of 10 – 20 repeating units and the core – a highly conserved oligosaccharide that connects the two (Figure 1). In order to make sense of the available immunological and structural data it is advantageous to align the repeating units of the O-antigen. The obvious way to do this is by aligning the biological repeating units (BRU). Not only does this allow internal epitopes to be recognized but the terminal residues, which are responsible for strong cross-reactivities between different serogroups, are also identified.

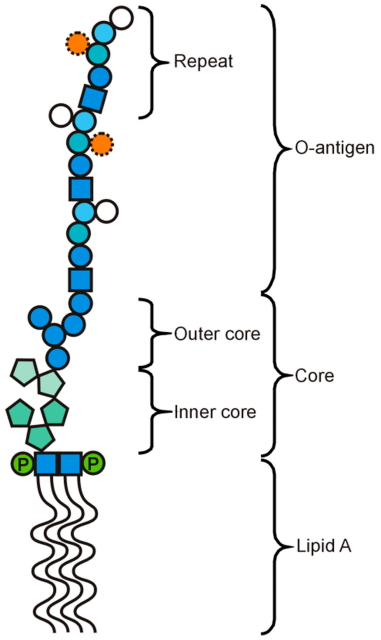
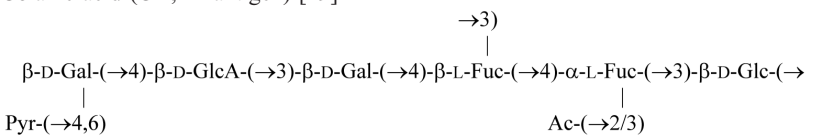


Figure 1. Schematic of a lipopolysaccharide

Unfortunately, there is little experimental data on the BRUs. It is however known that biosyntheses of the BRU commences with the transfer of a glycosyl phosphate to undecaprenylphosphate [1] (the synthesis of other polysaccharides, such as teichoic acids [4] and capsular polysaccharides [5], also starts with the same carrier). The glycosyl residue is most

often D-GlcNAc but other 2-acetamidodeoxy sugars and D-galactose [6] are occasionally used. While preparing a compilation of *E. coli* O-antigen structures we made several other observations that allowed an increasingly detailed set of rules to be derived. It should be noted, however, that most of these rules have exceptions and they are applicable only to polysaccharides that are made by the polymerisation of pre-formed oligosaccharide blocks (Wzy-dependent pathway) and not to those made by the ABC transporter-dependent pathway. In *E. coli*, both O-antigens, with few exceptions [3], as well as type 1 (former IA) and 4 (former IB) capsular polysaccharides (CPS, K-antigens) [7], enterobacterial common antigen (ECA) [8], and colanic acid (CA) [9] (Scheme 1) are formed by this pathway.

Colanic acid (CA, M-antigen) [19]



Enterobacterial common antigen (ECA)[18]

→3)-α-D-Fuc4NAc-(→4)-β-D-ManNAcA-(→4)-α-D-GlcNAc-(→

Scheme 1. Colanic acid and the enterobacteria common antigen – two cell surface polysaccharides common to most *E. coli* strains

Synthesis of Polysaccharides by the Wzy-dependent Pathway

In the Wzy-dependent pathway the repeating unit is built by the sequential transfer of glycosyl residues from sugar nucleotides to an undecaprenyl pyrophosphate linked sugar residue (Figure 2). In *E. coli*, as well as in many other bacteria, this first residue is linked to the lipid by WecA (undecaprenyl-phosphate α-*N*-acetylglucosaminyl 1-phosphatetransferase) in the Enterobacterial Common Antigen (ECA) synthesis and is a D-GlcNAc or D-GalNAc residue [1].

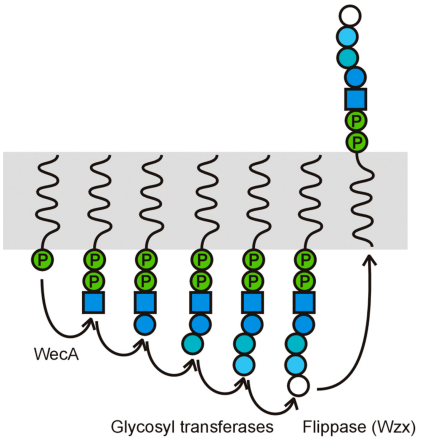


Figure 2. Formation of the biological repeating unit on the cytosolic side of the inner membrane

Salmonella on the other hand often uses D-Gal derived from the M-antigen synthesis as the first residue. Thus, only certain sugar residues are allowed in the first position of the repeating unit. Additional glycosyl residues are attached in a sequential manner. The first being added to the 3 position (rarely the 4 position) and the completed lipid bound oligosaccharide ''flipped'' to the periplasmic side of the membrane. This oligosaccharide is the biological repeating unit of the O-antigen. The protein responsible for the translocation is called ''flippase'' (Wzx). On the periplasmic side the oligosaccharides are joined by a polymerase (Wzy) causing the polysaccharide to become elongated from the ''reducing'' (lipid bound) end – hence the name of the pathway (Figure 3). Finally the O-chain is transferred to the lipid-A bound core by a ligase (WaaL) and exported to the outer surface of the outer membrane. Occasionally the oligosaccharide is modified in the periplasm by glycosidases and acetylases encoded by temperate phages [10]. Phages may also disrupt the normal polymerisation and change the linkage between BRU:s [11].

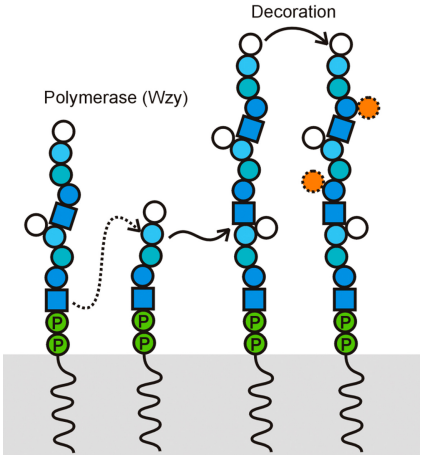


Figure 3. Polymerisation and decoration of O-antigen on the periplasmic side of the inner membrane

Note that the initial transfer of the glycosyl phosphate to the undecaprenylphosphate as well as the final transfer of the lipid bound polymer to the core oligosaccharide is achieved by enzymes encoded outside the O-antigen gene cluster, as are the glycosyltransferases and acetyltransferases that effect the modification of the polymer in the periplasmic space. It has however been reported that a branch is added by a glycosyltransferase in the O-antigen gene cluster [12]. The O-antigen gene cluster, which is most often, but not always, located on the chromosome contains all the genes necessary to make the needed nucleotide sugars (except UDP-Glc, UDP-Gal, and UDP-GlcNAc which are present in sufficient quantity anyway [13]), the glycosyltransferases for the formation of the linkages, and the proteins required for translocation (Wzx), polymerisation (Wzy) and for controlling the polymer length (Wzz). An elegant illustration of the biosynthesis is provided by an attempt to express an exopolysaccharide of *Streptococcus thermophilus* in *Lactococcus lacti* [14]. The polymer produced after transfer of the gene cluster had the proximal D-GalNAc residue replaced by D-Gal and the branch was absent (Scheme 2). Both the proximal residue, defined by the transfer of the first residue to the undecaprenyl phosphate carrier, and the decorating D-Gal residue are expected to be the result of enzymes encoded in other regions of the chromosome.

Streptococcus thermophilus Sfi6 (original)

α-D-Gal-(→6)
|
→3)-β-D-Gal-(→3)-β-D-Glc-(→3)-α-D-GalNAc-(→

Lactococcus lacti MG1363

→3)-β-D-Gal-(→3)-β-D-Glc-(→3)-α-D-Gal-(→

Scheme 2. Transferred O-antigen gene cluster
The proximal D-GalNAc is replaced by D-Gal and the branching residue is lost. [14]

Alignment of *E. coli* O-antigens

At first we simply aligned the sequences with D-GlcNAc at the right most position. It soon became apparent that D-GalNAc could replace D-GlcNAc and that extension at the 3-position was highly preferred. Also, branches at other positions than that joining the next BRU were often D-Glc or D-Gal residues – a situation similar to that in *Shigella flexneri*. In *S. flexneri* these additional glycosyl residues are added on the periplasmic side of the inner membrane by glycosyl transferases encoded by temperate phages [10]. Since it is rather uncommon to find O-antigens with more than one D-GlcNAc/D-GalNAc residue this alone was sufficient to assign the BRU in > 60% of the structures. The results of the alignments have been published [3].

Rules derived from *E. coli* O-antigens

Using our knowledge of the *E. coli* O-antigens it became possible to derive a set of rules to guide the assignment of BRUs in cases when there was more than one possible ''initiating'' residue. The rules also allow polysaccharides containing amino sugars but synthesised by the ABC-transporter pathway to be recognised as such (Figure 4).

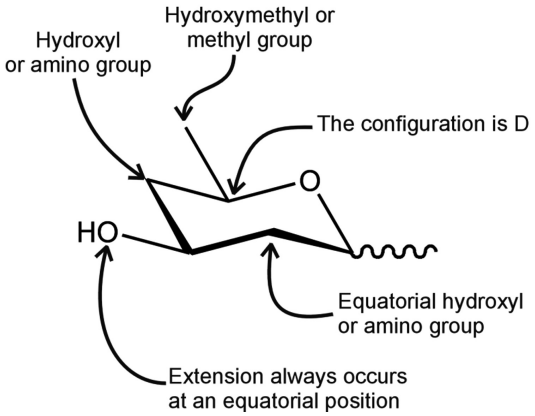


Figure 4. Common structural features in the first lipid bound residues.

Rule 1 – The first residue in the BRU is a 3-linked β-D-GlcNAc

On examining the structures of all the known *E. coli* O-antigen structures it was observed that the by far most common residue at the first position in the BRU is D-GlcNAc (72%). Occasionally this residue is α-linked or a D-GalNAc residue (25%) and in a few serogroups the linkage is to position 4 in D-GlcNAc. There are two structures (3%) where D-FucNAc replaces D-GlcNAc. It has been claimed that the occasional use of D-GalNAc instead of D-GlcNAc is due to a lack of selectivity in WecA, the enzyme that transfers the first residue to the lipid carrier [15]. Recent results however suggest that the lipid bound D-GlcNAc might be transformed by an epimerase [16]. This would allow the WecA enzyme to be selective, yet still provide a mechanism by which other lipid linked aminosugars may occupy the first position in the repeating unit. It is possible that both alternatives might be operative in different serogroups.

Rule 2 – The BRU is four or five residues long

The length of the repeating units does not vary greatly. The most common lengths for *E. coli* O-antigens are four (58%) and five (29%) and this appears to be common in other bacterial polysaccharides, too. Exopolysaccharides, e.g. those formed by lactic acid bacteria, tend to have longer repeating units [17]. It should also be noted that the enterobacterial common

antigen (three residues [18]) and colanic acid (six residues [19]) fall outside this range. Many carbohydrate epitopes, e. g. the blood group antigens, are three to four residues in size. Thus, a repeating unit consisting of four to five residues with one conserved residue and three to four variable residues may be optimal for interaction with proteins.

Rule 3 – Branches consisting of D-*Glc,* D-*Gal or* D-*GlcNAc residues are added after polymerisation*

Many O-antigen structures are branched. One way in which branches are formed is in the polymerisation of the repeating units. These branches are easily recognised since they can only occur at residue that is linked to the first residue on the next BRU, e. g. D-GlcNAc or D-GalNAc. There are several repeating units where there is more than one branch point, or where the branch point is located on a residue not linked to the next BRU. Most of these branches are D-Glc (50%), D-Gal (17%) or D-GlcNAc (20%) that may be added on the periplasmic side of the inner membrane by glycosyl transferases encoded by temperate phages. The genes coding for the transfer of these sugars to a lipid carrier, their translocation to the periplasmic side of the inner membrane and transfer to the growing polymer are located outside the locus for the main O-antigen genes [10]. It seems that this mechanism is restricted to nucleotide sugars readily available from housekeeping. The inactivation of these genes does not hinder LPS formation and has been suggested as a mechanism by which *Shigella* spp. may undergo phase variation [20]. The heterogeneity frequently claimed to exist in LPS seems to be linked to these residues or other substituents added after polymerisation of the oligosaccharide blocks. There is evidence that in some cases this 'heterogeneity' is not caused by non-stoichiometric substitution but by the presence of different polysaccharide chains of well-defined structure which occasionally can be separated [21, 22].

Rule 4 – Rare glycosyl residues are likely to occur as the last residue in the BRU

E. coli O-antigenes are made up of 25 different glycosyl residues, some more common than others. The common residues D-Glc, D-GlcNAc, D-Gal, D-GalNAc, D-Man and L-Rha each constitute more than 10% of all residues. Together they account for 77%. The remaining 19 residues make up the remaining 23%. Often these rare glycosyl residues are at the end of the BRU. This makes them particularly exposed. At the last position of the BRU they account for approximately 50%, i. e. they are twice as frequent as would be expected. Since the last residue in the BRU is also expected to be the most exposed one it is not surprising that it should also show the greatest variation.

Rule 5 – O-antigens with repeating units violating the above rules are derived from the ABC-transporter dependent pathway

There are two other known pathways for O-antigen synthesis; the ABC-transporter dependent pathway and the synthase dependent pathway. The latter has so far only been encountered in *Salmonella borreze* [23]. In the ABC-transporter dependent pathway the polymer is formed on the cytosolic side of the inner membrane by addition of one glycosyl residue at a time. The completed polysaccharide is then translocated by an ABC-transporter. Often the number of different residues is small and the backbone short. Slightly less than 10% of the *E. coli* O-antigens are made by this pathway [3].

Correlation of Structure with Pathotype

Since there is a correlation between serotype and pathotype it is obvious to seek similarities in the corresponding O-antigen structures. Serogroups containing pathogenic strains often harbour strains belonging to more than one pathotype and may contain commensal strains as well. Mimicry of host glycans has been proposed to be related to virulence in several genera of bacteria but in *E. coli* mimicry seems to be marginal or even coincidental. The impression is strengthened when the rather low similarity of the antigens to mammalian glycans is contrasted with the elaborate mimicry and phase variation encountered in the lipooligosaccharides of *Helicobacter pylori* and *Campylobacter jejuni* [24]. However spontaneous serotype conversion has been observed in *Shigella* and may be related to the persistence of infection [25].

Mimicry of blood group antigens seems to occur in some serogroups of the EHEC (enterohaemorrhagic *E. coli*) pathotype. The blood group mimicked is H (B in O86) which is the most common blood group globally but also the simplest structure. When examining a larger set of O-antigens related to EHEC one finds that α-6-deoxy-L-hexoses (α-Col, α-L-Fuc, α-L-Rha) are located at the terminal position in most structures (65%) and that amino sugars are quite common (77% contain more than one). The lack of fidelity, only the outermost part of the BRU is involved, makes it unlikely that any lasting protection from the immune system is achieved but this does not rule out a role in morbidity, e.g. by induction of autoimmune responses against similar antigens. Bacterial infections are known to play a role in autoimmune disorders such as Guillain-Barré syndrome (GBS) or Miller Fisher syndrome (MFS) [26]. Of the approximately 70 know O-antigen structures of *E. coli* only five display terminal blood group antigens and two more contain terminal α-Neu5Ac.

LPS is often claimed to be a virulence factor, but this is often based on the avirulence of strains deficient in LPS – a condition known to make the bacteria sensitive to serum and impair the function of the outer membrane. It has been proposed that glycosylation increases the virulence of *Shigella* by changing the conformation of the polysaccharide making the LPS layer more compact and thereby better exposing virulence factors on the cell surface

[27] but convincing proof that the actual structure of the O-antigen plays a role in virulence has yet to be presented. Recent results on the correlation between serogroup and the presence of toxins and adhesins are also starting to cast doubt on the correlation between pathology and serology. Thus in one study only 23% of the "pathogenic" *E. coli* strains identified by serology did also have the genes necessary to produce toxins although in some serogroups the correlation was much better (e.g. 70 – 80% in O111 and O119) [28]. The role of LPS structure in disease is further obscured by the presence of O-antigen gene clusters on plasmids that also carry virulence factors such as adhesins and toxins.

Serological Cross Reactivity and Conserved Structural Motifs

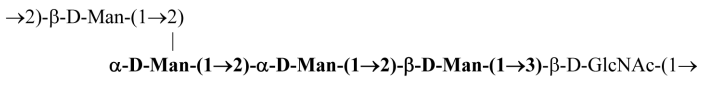
There is extensive cross-reactivity between different strains of *E. coli* but also between *E. coli* and other genera. In many cases the O-antigens are identical or near identical. The similarity between these polysaccharides becomes apparent only when the terminal residues of the biological repeating unit are considered. If the branches, assumed to be added by phage encoded transferases, are removed and the first residue of the BRU is disregarded, one finds that there are some motifs that are shared between many more serotypes and genera.

In the case of *E. coli* O123/*S. enterica* O:58 [29] and *E. coli* O17, O44, O73, O77 and O106/*S. enterica* O:6,14 [30] analysis of the O-antigen gene clusters suggest that they originate from a common ancestor (14×10^7 yrs ago). In the case of *S. sonnei* the O-antigen cluster located on the plasmid seems to be acquired fairly recently ($8 - 10 \times 10^3$ yrs ago) from *P. shigelloides* O17 [31]. The original chromosomal O-antigen cluster (*E. coli* O7 or O62[32]) was subsequently lost. Thus there seem to be both ancient structures as well as some of recent origin. Much of the diversity of the O-antigens seems to be related to phage induced changes on highly conserved structures. Three structural motifs that are wide spread are the Man_3-motif of *Salmonella thompson* (Scheme 3), Rha_3-motif of *Shigella flexneri* Y (Scheme 4), and the Col-Gal-GlcNAc-motif of *Escherichia* O55 (Scheme 5). No doubt many more such "clusters" of similar structures are still waiting to be discovered.

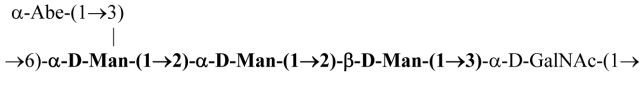
Salmonella cerro [36], *Citrobacter freundii* O23 [37]

→4)-α-**D-Man-(1→2)-α-D-Man-(1→2)-β-D-Man-(1→3)**-α-D-GalNAc-(1→

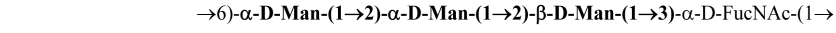
Salmonella thompson [38]



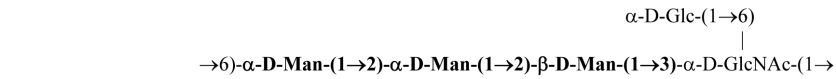
Yersinia pseudotuberculosis O:2c [39]



Enterobacter cloacae O10 [40]

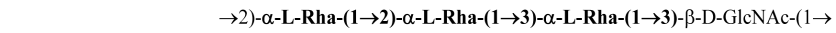


Escherichia coli O17 [41]

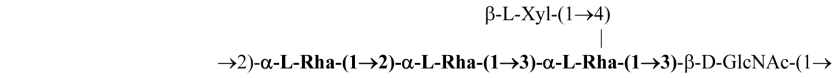


Scheme 3. O-antigens containing a α-D-Man-(1→2)-α-D-Man-(1→2)-β-D-Man motif.

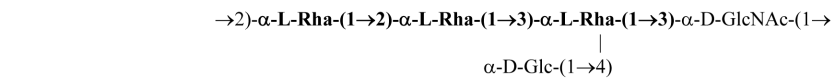
Shigella flexneri Y [42]



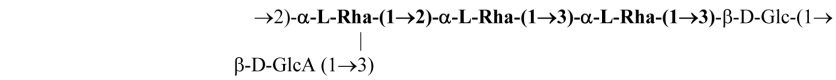
Pseudomonas solanacearum ICMP 8072 [43]



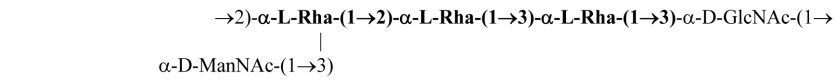
Serratia marcescens O10 [44]



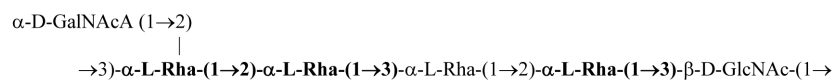
Klebsiella K45 [45]



Acinetobacter baumannii O10 [46]



Salmonella arizonae O62 [47]

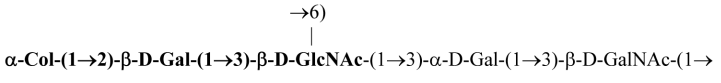


Scheme 4. Bacterial polysaccharides containing a α-L-Rha-(1→2)-α-L-Rha -(1→3)-α-L-Rha motif

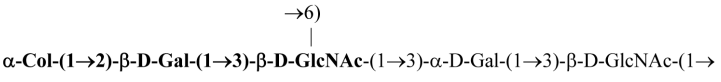
Whilst studying a wider range of repeating unit polysaccharides it also became apparent that several other amino sugars could replace D-GlcNAc as the first residue of the BRU. In *E. coli* we found D-GalNAc as well as D-FucNAc but in other genera D-QuiNAc, D-FucNAc4NAc and D-QuiNAc4NAc (Bacillosamine) can take its place. Thus the common requirement seems to be a D-hexosamine with equatorial substituents at position 2 and 3. In CPS and

EPS D-GlcNAc is generally replaced by D-Gal or D-Glc. Extension proceeds at an equatorial position, hence a few cases of 4-linked D-GlcNAc are encountered but all other residues link through position 3.

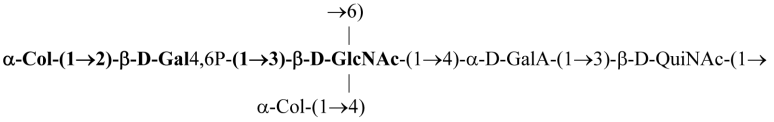
Escherichia coli O55 [48]



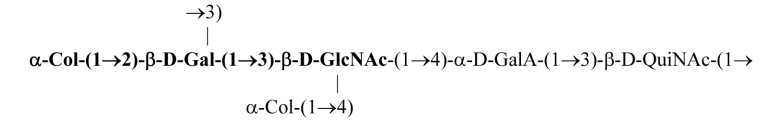
Salmonella arizonae O50 [49]



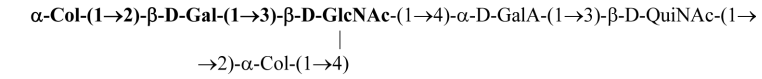
Vibrio cholerae O139 [50]



Aeromonas trotta [51]



Pseudoalteromonas tetraodonis IAM 14160^{T} [52]



Scheme 5. O-antigens containing a α-Col-(1→2)-β-D-Gal-(1→3)-β-D-GlcNAc motif

SUMMARY

The structures of the repeating units of *E. coli* lipopolysaccharides where aligned to give the best agreement with known biosynthetic pathways of the biological repeating units. During this process additional rules could be derived that allowed extension to other genera and types of polysaccharides made by the polymerase dependent pathway. The comparison of the BRUs of polysaccharides from different genera of bacteria showed that common motifs are fairly widespread. Although O-antigens are considered to be very variable genetic studies show that some BRUs originate from common ancestors several million years ago. Mimicry of host glucans is often claimed for polysaccharides but mimicry is rare and many

backbones appear to be older that the hosts that bacteria inhabit. It would appear that there are other evolutionary forces at work, perhaps microbe-microbe rather than microbe-host interactions.

Although there are many reports of heterogeneity in lipopolysaccharides the fidelity of O-antigen synthesis seems to be high and under strict temporal and/or spatial control. Despite the fact that several enzymes in the pathway seem to be quite promiscuous the resulting polymers are often well defined.

The alignments allow easy identification of common epitopes and can be used to determine the biosynthetic pathway for a given structure. In some cases it is also possible to determine the reliability of published structures. As more structures are compared it will hopefully be possible to rationalize more of the polysaccharide structures and perhaps derive additional rules. Amongst other, it was observed that some residues show a high preference for certain positions in the BRU, e.g. some residues are always found in terminal positions whereas others never are.

Whilst exploring carbohydrate structures extensive use was made of glycomics databases; CCSD (accessed through the interface at http://www.boc.chem.uu.nl/static/sugabase/carbbank.html) [33], GLYCOSCIENCES.de (http://glycosciences.de) [34], and BCSDB (http://www.glyco.ac.ru/bcsdb3/) [35]. Better integration, not only of available structure and genetic data is needed but also a much more multidisciplinary approach to glycoscience in general. Much remains to be discovered, in particular with regard to the genetic data that is starting to appear. In a not too distant future one can envision the both the possibility to tailor bacterial polysaccharides for industrial or medical applications as well as a better understanding of the biological role of lipopolysaccharides.

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