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Chapter 13

BACTERIAL CAPSULAR POLYSACCHARIDES OF PATHOGENS – A TOOLBOX FOR VACCINES AND THERAPEUTICS

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ABSTRACT

The bacterial capsule is a hydrated polysaccharide structure that covers the outermost layer of the cell wall. It is an important virulence factor and acts as armor in shielding the bacteria from a variety of environmental pressures and host immune defenses. Considerable structural diversity exits not only between capsular polysaccharides of different bacterial species, but also within the same species. While most pathogenic bacteria are encapsulated, most encapsulated bacteria are not pathogenic. As a result, understanding the structural and immunological diversity of capsules together with cellular components and machinery involved in capsule biosynthesis is paramount in developing new therapeutics to fight deadly bacterial infections. This chapter presents an overview of the capsular polysaccharide of pathogenic bacteria. This overview includes the structural diversity of capsules among virulent bacteria, the organization of capsule genetic elements, the mechanisms of capsule biosynthesis and transport, along with current technologies employed in the preparation of glycoconjugate vaccines.

Keywords: bacterial virulence, capsular polysaccharide, K-antigen, capsule diversity, gram-negative bacteria, gram-positive bacteria, capsular gene organization, capsular biosynthesis, ABC transporter pathway, wzy pathway, synthase pathway, capsule transport, glycoconjugate vaccines, vaccine preparation technologies

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ABBREVIATIONS

ABC	ATP binding cassette
ATP	Adenosine triphosphate
BCSDB	Bacterial Carbohydrate Structure Database
CMP	Cytidine monophosphate
CPS	Capsular polysaccharides
CRM197	Non-toxic mutant *Corynebacterium diphtheriae* toxin
GAG	Glycosaminoglycans
GAS	Group A streptococcus
GBS	Group B streptococcus
GDP	Guanosine diphosphate
GlcNAc	*N*-acetylglucosamine
GlcUA	glucuronic acid
GT	Glycosyl transferase
HA	Hyaluronic acid
HAS	Hyaluronan synthase
KDO	3-deoxy-D-mann-octulosonic acid
LPS	Lipopolysaccharides
NGT	*N*-glycosyltransferase
NmB	*Neisseria meningitidis* serogroup B
NmC	*Neisseria meningitidis* serogroup C
OMP	Outer membrane protein PFCSDB, Plant & Fungal Carbohydrate Structure Database
dTDP	2'-deoxy-thymidine-5'-diphosphate
TM	Transmembrane
Und-P	Undecaprenol phosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate

1. INTRODUCTION

The surfaces of bacteria are coated with glycans that play multiple roles in the interaction of these organisms with their environment. Bacteria have a very diverse assortment of glycans on their surfaces. This variety includes; diversity in monosaccharide composition, types of polymer structures, molecular size and number of chains. Most bacteria are coated with multiple glycan structures (Figure 1). The roles that these glycan play include but are by no means limited to: structural integrity, protection against host defense mechanisms, survival under adverse conditions such as heat and lack of water, symbiosis, and controlling the flow of nutrients [1]. It has been demonstrated that some bacteria in the gut microbiome produce glycans as signals for the host or for other bacteria to produce needed nutrients. The foundation for the structural integrity of most bacteria is a highly crosslinked peptidoglycan whose structure and physical composition varies with the genus [2]. The outer leaflet of the outer membrane of Gram-negative bacteria consists of a lipopolysaccharide (LPS) (Figure 1). The

LPS is part of a protective barrier shielding the bacteria against dangerous environmental compounds. In pathogenic Gram-negative bacteria, LPS is also an important virulence factor exhibiting toxicity commonly referred to as endotoxin. The surface of *Mycobacteria* is more complex, and in addition to several glycans has an outer lipid bilayer consisting of lipoglycans. Mycobacteria are intracellular pathogens and have developed a protective barrier of glycans and lipoglycans well suited to resist the defenses of eukaryotic host cells [1]. In this chapter we present an overview of one of the surface glycans of bacterial pathogens, namely, capsular polysaccharide. Our overview includes; structural diversity, organization of gene clusters encoding synthesis, biosynthesis and assembly, and use of these polymers to prepare vaccines and therapeutics. This chapter is not intended to be comprehensive, since there are several in depth reviews on the structure, biosynthesis, and use of bacterial capsular polysaccharides available to the reader [3-7].

2. Capsular Polysaccharides of Pathogenic Bacteria

While the human microbiome consists of a vast number of bacteria species, a small number of these species are pathogens. Many of these pathogens are coated with capsular polysaccharides which are extracellular to most other surface structures and thus are first to encounter the environment (Figure 1). Thus, capsular polysaccharides can serve as a physical barrier to the host immune system. Early investigators of infectious disease showed that immunization of animals with encapsulated bacteria results in the formation of antibodies specific for the capsule. The specificity of these antisera was used to group these organisms into serogroups or serotypes [7]. Subsequently, it was shown that the chemical structure of these capsular polysaccharides defines the serogroups [5, 6]. Epidemiologist have used these serogroups as tools to determine which strains are associated with disease and the prevalence of disease-causing serogroups in specific geographic regions. For example, there are 6 serotypes of *Haemophilus influenzae* based on capsular polysaccharides, but most of the disease due to *H. influenzae* was caused by type b at the time of licensure of the first vaccine against this organism. Similarly, there are 13 capsular groups of *Neisseria meningitidis* based on their capsular polysaccharides, but most of the disease globally is caused by 6 serogroups, namely, ABCWX and Y. The distribution of disease associated with a serogroup varies geographically and temporally. Antibodies raised against the capsule often affords protection against disease caused by infection with a specific serogroup. This forms much of the basis for the development of polysaccharide-based vaccines.

The number of different capsular polysaccharides associated with a given species can vary widely, from over 90 different structures identified for *Streptococcus pneumoniae* to only 1 polysaccharide structure for *Streptococcus pyogenes* or *Salmonella typhi*. Interestingly, although there may be many structures or serogroups associated with a species, only a select few of these serogroups are associated with most of the disease caused by that species. Polysaccharide structures were originally identified by assays using specific antibodies and hence classified into serogroups. The evolution of chemical and physicochemical methods for analysis and detection of polysaccharides has helped establish the chemical basis for differences and relatedness of serogroups. The early work of Michael Heidelberger [8] defined the serology of a large number of structures on *Streptococcus pneumoniae*. The structure of

most of these polysaccharides have been determined and include all serotypes most commonly associated with disease. A list of some of these structures is listed in Table 1. Capsular polysaccharides are usually long chains of repeating unit oligosaccharides. The repeating unit oligosaccharides of *S. pneumoniae* can be acidic or neutral, branched or linear, and substituted with non-carbohydrate substituents that often influence antigenicity. The Seeberger group has developed a solid phase approach to synthesize the repeat units of the pneumococcal polysaccharides [9, 10]. These investigators use this synthetic approach to define antigenically important structures in repeat units and the antigenically relevant sequence of the repeating structure in pneumococcal polysaccharides.

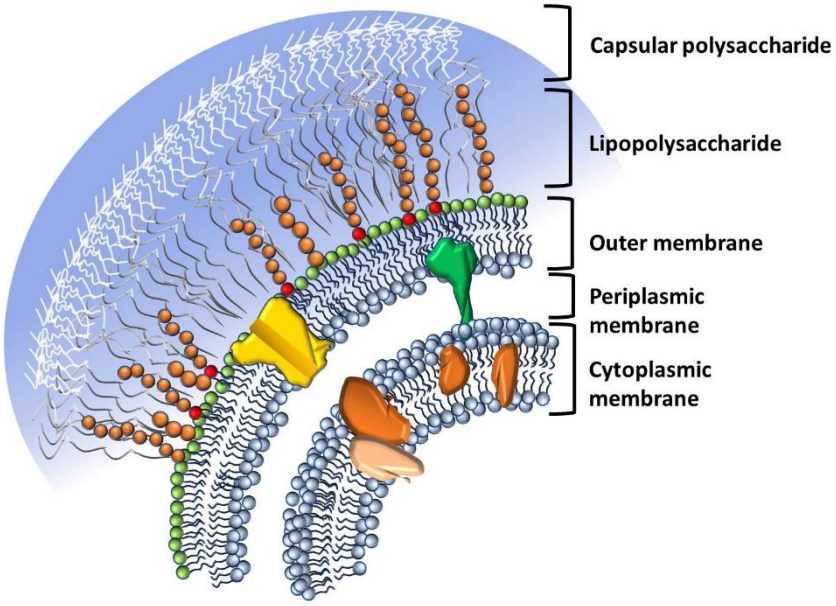


Figure 1. A schematic illustration of a Gram-negative bacterial cell surface.

Table 1. Structures of capsular polysaccharides from multiple Gram-positive bacteria. Serogroups 7A, 14 and 37 in *Streptococcus pneumoniae* are neutral in charge. Serogroup 1 in *Streptococcus pneumonia* is zwitterionic and the remaining polysaccharide structures are anionic. Serogroups 18F and 27 in *Streptococcus pneumoniae* carry non-carbohydrate substituents in the form of glycerol, pyruvate and choline

Organisms	Serogroups /Serotypes	Structures of repeating units	References
Streptococcus pneumoniae	1	→3)-α-AATGal*p*-(1→4)- α-D-Gal*p*A$2_{0.3}$,$3_{0.3}$$Ac_2$-(1→3)-α-D-Gal*p*A-(1→	[18]
	3	→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→	[8]
	7A	→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*N 4 ↑ 1 α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*	[8]

Organisms	Serogroups /Serotypes	Structures of repeating units	References
	14	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ 1 β-D-Gal*p*	[8]
	18F	Gro(1→P ↑ 3 →4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*2Ac-(2 ↑ 1 α-D-Glc*p*6Ac	[8]
	19F	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→P→	[8]
	27	→3)-β-D-GlcpNAc4,6(*S*)Pyr-(1→3)-α-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→4)- 2 ↑ Cho→P	
	37	→3)-β-D-Glc*p*-(1→ 2 ↑ 1 β-D-Glc*p*	[19]
S. pyogenes		→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→	[20]
S. agalactiae	1A	→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→ 3 ↑ 1 α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc	[17]
Staphylococcus aureus	5	→4)-β-D-Man*p*NAcA-(1→4)-α-L-Fuc*p*NAc3Ac-(1→3)-β-D-Fuc*p*NAc-(	[17]
	8	→4)-β-D-Man*p*NAcA4Ac-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Fuc*p*NAc-(	[16]

AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galatose; Ac, acetate; Cho, choline; FucNAc, *N*-acetylfucosamine; Gal, galactose; GalA, galacturonic acid, GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; Gro, glycerol; ManNAc, *N*-acetylmannosamine; ManNAcA, *N*-acetylmannuronic acid; Neu5Ac, *N*-acetylneuraminic acid; Pyr, pyruvate; Rha, rhamnose; *f*, furanose; *p*, pyranose.

Over 80 serologically distinct capsular polysaccharide (K-antigens) serotypes have been identified in *Escherichia coli* [11] which, like *S. pneumoniae*, vary widely in complexity and chemical structure. However, only a few serotypes of *E. coli* are frequently associated with disseminating infections. These serotypes are encapsulated with polysaccharides having acidic monosaccharide or disaccharide repeating units (Table 2). This contrasts with the diversity of structures associated with strains that account for most of pneumococcal disease. This theme of acidic and simple repeating structures of encapsulated predominant disease isolates is also seen with *Haemophilus influenzae*, *Neisseria meningitidis*, and *Staphylococcus aureus* (Table 1 and Table 2). The majority of *Haemophilus influenzae* associated with disease such as meningitis and otitis media are encapsulated with serotype b, a ribosylribitol phosphate polysaccharide. Most meningococcal disease is caused by *N. meningitidis* groups A, B, C, Y, W, and X. Groups A and X polysaccharides are homopolymers of a *N*-acetylhexosamine phosphates and B and C are polysialic acids. Groups W and Y polysaccharide repeat units are disaccharides of hexose and sialic acid (Table 2). Over 75% of clinical isolates *Staphylococcus aureus* have type 5 and type 8 capsules [12-15]. These *S. aureus* capsular polysaccharides have trisaccharide repeating units containing the unusual monosaccharides N-

acetylmannosaminouronic acid, L-*N*-acetylfucosamine and D-*N*-acetylfucosamine (Table 1) [16]. This simplicity in structure is repeated with several other pathogens such as *Salmonella typhi* and *Streptococcus pyogenes.* The *S. typhi* Vi antigen is a homopolymer of *N*-acetylgalactosaminouronic acid. This polysaccharide is partially *O*-acetylated. As is often the case with capsular polysaccharides of bacterial pathogens, the *O*-acetyl group is an important part of the antigenic epitope. *Streptococcus pyogenes* produces a capsule consisting of hyaluronic acid [17], a repeating structure of the disaccharide -4)-β-GlcUA-(1-3)-β-GlcNAc- (Table 1).

With the increasing number of bacterial genomes available more potentially encapsulated bacteria have been identified. A database of bacterial polysaccharide structures exists that contains many of the confirmed capsular polysaccharide structures. This data base was first established in 2005 (F. V. Toukach, Y. A. Knirel "New database of bacterial carbohydrate structures", Glycoconjugate J, 2005, 22: 216-217.) The Bacterial (BCSDB) and Plant & Fungal (PFCSDB) databases were merged in 2015 to improve the quality of content-dependent services, such as taxon clustering or NMR simulation. The separate databases will be supported in parallel until 2020.

Table 2. Structures of capsular polysaccharides from multiple gram-negative bacteria

Organisms	Serogroups/Serotypes	Structures of repeating units	References
E. coli	1 (and *N. meningitidis* Serogroup B)	→8)-α-D-Neu*p*5Ac-(2→	[21, 22]
	4	→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→ 3 ↑ 2 β-D-Fru*f*	[23]
	5	→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1→	[24]
	12	→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→5)-β-D-KDO*p*7/8	[25]
	30 (and *Klebsiella* K20)	→2)-α-D-Man*p*-(1→3)-β-D-Gal*p*(1→ 3 ↑ 1 β-D-Glc*p*A-(1→3)-α-D-Gal*p*	[5]
	92	→8)-α-D-Neu*p*5Ac-(2→9)-α-D-Neu*p*5Ac-(2→	[26]
H. influenzae	b	→3)-β-D-Rib*f*-(1→1)-D-RibOH-(5→P →	[27]
N. meningitidis	A	→6)-α-D-Man*p*NAc3Ac-(1→P →	[28]
	C	→9)-α-D-Neu*p*5Ac7/8Ac-(2→	[22]
	L	→3)-β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-G	[29]
	W	→3)-α-D-Gal*p*-(1→4)-α-D-Neu*p*5Ac-(2→	[30]
	X	→4)-α-D-Glc*p*NAc-(1→P →	[28]
	Y	→6)-α-D-Glc*p*-(1→4)-α-D-Neu*p*5Ac7/9Ac-(2→	[30]

Ac, acetate; Fru, fructose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; KDO, 2-keto3-deoxy-D-mannooctulonic acid; ManNAc, *N*-acetylmannosamine; Neu5Ac, *N*-acetylneuraminic acid; Rib, ribose; RibOH, ribitol; *f*, furanose; *p*, pyranose.

3. ORGANIZATION OF GENES ENCODING CAPSULAR POLYSACCHARIDE BIOSYNTHESIS

The genes responsible for the synthesis and export of bacterial capsular polysaccharides are organized in specific clusters. The group 2 gene clusters of *E. coli* were the first capsular polysaccharide genes cloned and expressed in a non-encapsulated bacterium [31, 32]. Today, whole genome sequences of many pathogenic bacterial strains are available facilitating more detailed descriptions of the genetic organization of genes encoding the machinery required for synthesis and export of bacterial capsular polysaccharides to the cell surface. In this section we present an overview on the organization of these genes.

3.1. Capsular Gene Clusters in Gram-Negative Bacteria

Polysaccharide capsule synthesized in Gram-negative bacteria needs to traverse a cytoplasmic membrane, periplasm, and outer membrane to reach the outer cell surface. Gram-negative bacteria predominantly use ATP binding cassette (ABC) or Wzy-dependent pathways to accomplish this task. Gene clusters in both these pathways are arranged with highly variable, capsule specific genes, flanked by conserved genes responsible for synthesis of sugars, transport and assembly of polysaccharide across the membranes. Of all the capsular gene clusters, *E. coli* is the most studied and is regularly used as a model system to compare other Gram-negative species (*Klebsiella pneumoniae*, *Haemophilus influenza*, *Neisseria meningitidis*, etc.).

3.1.1. Escherichia coli

The serogroups of *E. coli* are classified into 4 different groups [33]. Groups 1 and 4 capsules utilize the Wzy-dependent pathway, while groups 2 and 3 make use of the ABC transporter dependent pathway.

3.1.1.1. Group 1 and 4 Capsules in *E. coli*

The genes required for the synthesis and translocation of capsule in Group 1 and 4 are located upstream to the *his* operon in the *E. coli* chromosome. This region is also shared by the genes responsible for colanic acid and O-antigen biosynthesis [34]. The general backbone architecture of the genes in the *his*-linked region the of *E. coli* chromosome starts with colanic acid biosynthetic genes on the 5' end followed by various housekeeping genes, such as, *galF* (encoding UTP-glucose-1-phosphate uridylyltransferase), *gnd* (6-phosphogluconate dehydrogenase), *ugd* (UDP-glucose 6-dehydrogenase) and *wzz* (O-antigen chain length determination protein). K-antigen related genes in group 1 and 4 are often found inserted in-between *galF* and *gnd* (Figure 2). The 5' end of the K-antigen gene locus (next to *galF*) in group 1 is defined by four conserved genes *wzi*, *wza*, *wzb* and *wzc* that encode trans-membrane capsule transport machinery [35, 36]. This region continues into a highly variable, serogroup specific set of genes encoding sugar nucleotide synthases, glycosyltransferases, a Wzy polymerase and a Wzx flippase [37]. For a more detailed description of group 1 and 4 gene organization, please consult the excellent reviews of Whitfield [34] and Reid et al. [38].

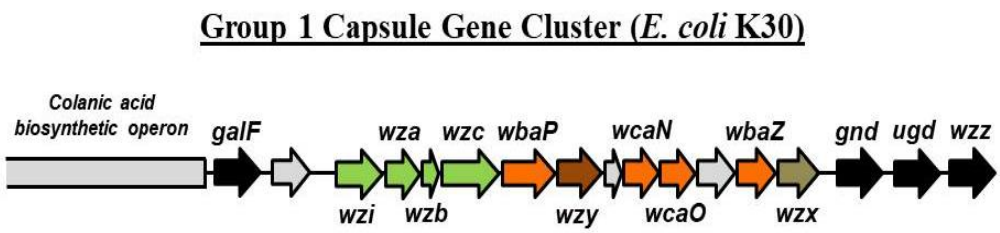


Figure 2. Organization of genes in capsular gene cluster of Gram-negative bacteria that use Wzy-dependent pathway. Group 1 and 4 *E. coli* capsule clusters and *Klebsiella pneumoniae* clusters fall under this category. The 5' end of the gene cluster starts with 4 conserved genes (colored in green) that are involved in trans-membrane capsule transport. This region followed by a less conserved set of genes (labeled orange, brown and tan) that encode for serotype specific proteins. The polymerase (wzy) and the flippase (wzx) are colored brown and tan respectively. Housekeeping genes that form the boundaries of gene cluster are colored in black.

3.1.1.2. Group 2 capsules in *E. coli*

Group 2 capsule producing gene clusters (*kps*) from *E. coli* K1 and K5 are considered as prototypes for studying the ABC transporter dependent pathway in many Gram-negative bacteria. *E. coli* K1 was the first capsular gene cluster to be cloned and expressed [37]. The gene cluster is located at min 64 of *E. coli* K12 chromosome, in a region between *serA* and *pheV* gene markers [7, 39]. The Group 2 gene cluster is subdivided into 3 regions (Figure 3A). Regions 1 and 3, that flank Region 2 on either side, are conserved across *E. coli* that produce group 2 capsules. Genes in this region are involved in initiation of capsule synthesis from the glycolipid anchor, and the translocation of fully synthesized capsule across the membranes [40, 41]. Region 2 on the other hand, encodes for glycosyltransferases and sugar nucleotide synthases that are K-antigen specific [42].

Transcription of Region 1, starting from PR1 promoter (σ70 family) produces a polycistronic mRNA that encodes for six proteins-KpsFEDUCS, some of which are still being investigated to determine their function [43]. Transcription of the rest of the gene cluster starts at PR3, located upstream to the region 3 and proceeds to the end of region 2 [44, 45]. *kpsMT* genes in region 3 are involved in the transport of capsular polysaccharide across the membrane. Transcription promoter PR1 and PR3 are sensitive to temperature and are non-functional below 20°C making thermal regulation a unique feature of group 2 capsule biosynthesis [46].

Variations to the order of gene arrangement into three distinct regions have been observed in *E. coli* K15 capsular gene clusters [47] (our unpublished work). The overall arrangement resembles that of a group 2 capsular gene cluster, but with only five genes (*kpsFEDU* and *C*') transcribed as a single unit (instead of six in group 2) in Region 1. *kpsC*' was found truncated and is not essential for capsule biosynthesis. Instead, *kpsC* and *S* genes, essential for capsule biosynthesis were located on the complimentary strand, downstream of region 2 along with five other capsule specific open reading frames (ORF's). In addition to this, the essential *kpsCS* are highly homologous to corresponding genes of group 3 capsules.

3.1.1.3. Group 3 Capsule in *E. coli*

E. coli K10 (Figure 3B) and K54 capsular gene clusters have been thoroughly investigated and are considered prototypes in explaining the genetics of group 3 capsule [48]. The overall gene organization into three regions and the location of the gene locus on the *E. coli* genome

are analogous to that of group 2. Although, regions 1 and 3 still constitute genes involved in the initiation of capsule synthesis to the glycolipid anchor and the transport of fully synthesized capsule across the membranes, they are rearranged between the regions. Region 1 and 3 of group 3 encode KpsD, KpsM, KpsT, KpsE KpsC, and KpsS respectively, and are homologues of similarly named proteins in group 2. Region 2 in group 3 is serotype specific and it separates regions 1 and 3, like the arrangement found in group 2 [49]. *kpsF* and *kpsU*, known to be involved in CMP-KDO biosynthesis are absent in group 3. It is suggested that *E. coli* with group 3 capsules, make use of a CMP-KDO synthase homologue, KdsB [50], from the lipopolysaccharide biosynthetic machinery to synthesize KDO for capsule synthesis.

3.1.2. Other Gram-Negative Bacteria

The serotypes in *K. pneumoniae* are as diverse as in *E. coli.* A total of 79 serotypes have been identified and gene clusters compared [51-53]. The general architecture of the gene cluster in *K. pneumoniae* is a direct match to the group 1 capsule gene cluster in *E. coli*. The entire gene cluster lies in between *galF* and *ugd* on the *K. pneumoniae* genome. The conserved set of genes include *orf2* (*cpsACP*), *wzi*, *wza*, *wzb*, *wzc*, and *gnd* from 5' to 3' end of the gene cluster. The regions in between *wzc-gnd* and *gnd-ugd* are filled with genes that are serotype specific and therefore are least conserved. In case of *wzc-gnd* region, the genes encode for Wzy polymerase, Wzx flippase, glycosyltransferases, sugar modifying enzymes, etc. while, *gnd-ugd* region contains genes *manCB* and *rmlABCD* that are involved in GDP-D-mannose and dTDP-L-rhamnose synthesis respectively [53]. Presence of these genes in *gnd-ugd* region always corresponds to capsules containing mannose and rhamnose in their repeat units. A few exceptions/variations to this general gene architecture is well documented in the literature elsewhere [52].

Haemophilus influenzae is responsible for a wide variety of infections ranging from bronchitis to meningitis. A total of six capsule serotypes (a to f) have been identified [54]. The capsule gene cluster in *H. influenzae* is usually found flanked between IS*1016* gene elements or downstream to *sodC* gene on the genome [55]. The arrangement of genes in *H.* influenzae is similar to the *E. coli* group 3 capsule gene cluster. It is composed of three distinct regions. Regions 1 and 3 each contain 4 and 2 genes designated *bexDCBA* and *hcsAB* respectively [56, 57]. Proteins transcribed from *bexA*, *bexB*, *bexC*, *hcsA* and *hcsB* are homologues of KpsT, KpsM, KpsE, KpsC and KpsS respectively. BexD is described as on outer membrane protein (OMP) that does not share homology with KpsD [49]. Genes in region 1 and 3 are involved in regulation and transport of polysaccharide across the membranes and therefore are conserved among serotypes. Genes in region 2 encode capsule type a- through f-specific proteins. A unique feature of *H. influenzae* capsule gene cluster is its ability to be amplified into multiple copies [58]. Capsulation in *H. influenzae* is highly unstable. To overcome this some highly virulent strains of *H. influenzae* serotype b, make multiple copies of its capsular gene cluster. This amplification happens only with gene clusters flanked by IS*1016.* Bacterial strains with more copies of capsular gene cluster are found with increased concentration of capsular polysaccharide on their membranes [59].

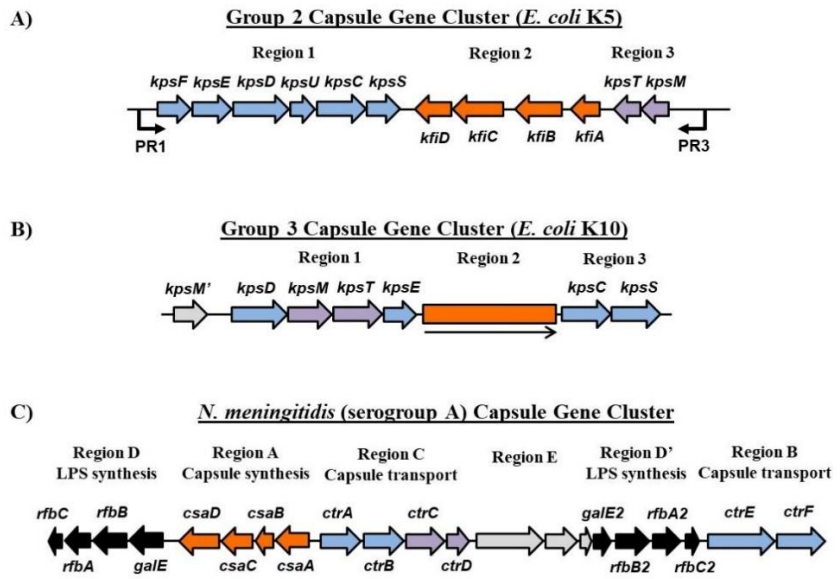


Figure 3. Organization of genes in capsular gene clusters of gram-negative bacteria that use the ABC transporter dependent pathway. *Haemophilus influenzae*, *Neisseria meningitidis*, group 2 and 3 *Escherichia coli* clusters are in this category. Highly conserved genes involved in the initiation of capsule synthesis and translocation across the membrane are colored in blue and purple. *kpsM*/*ctrC* and *kpsT*/*ctrD* are translated into homologous proteins (purple). Regions colored orange contain genes that are serotype specific.

N. meningitidis is another Gram-negative, highly virulent bacteria that uses the ABC transporter dependent pathway, with homology to group 2 and 3 capsule producing *E. coli* stains. Out of 13 known serotypes, 6 (A, B, C, W, X and Y) are known to be invasive. The capsule gene cluster in *N. menigitidis* is complex compared to other Gram-negative species. It consists of six region, D-A-C-E-D' and D, in that particular order (Figure 3C). While regions D and D' are involved in LPS biosynthesis, the function of region E is still unknown [60]. Of the remaining 3 regions, capsule specific genes are encoded within region A, followed by capsule synthesis initiator and translocation genes placed within regions C and B. Region C encodes for a total of four capsule transport genes *ctrABCD* that are organized similar to region 1 of group 3 capsule producing *E. coli.* Like in *H. influenzae*, three proteins encoded in this region share homology with translocation proteins in *E. coli* (CtrA/KpsE, CtrC/KpsM and CtrD/KpsT) except for CtrB, which is an outer membrane protein [49, 61]. Genes *ctrE* and *F* from region B are homologs of *E. coli* gene *kpsC* and *S*, respectively [62].

3.2. Capsular Gene Clusters in Gram-Positive Bacteria

Gram-negative species primarily use Wzy and Synthase-dependent mechanisms for synthesis and transport of polysaccharides across the membrane. The diversity of polysaccharide structures among various Gram-positive species is quite notable. The most common encapsulated Gram-positive human pathogens are *Streptococcus pneumoniae*, *Streptococcus agalactiae* (group B streptococcus, GBS), *Streptococcus pyogenes* (group A streptococcus, GAS) and *Staphylococcus aureus.*

3.2.1. Streptococcus Pneumoniae

Of more than 90 serotypes of *S. pneumoniae* identified, all except two serotypes (serotypes 3 and 37) make use of the Wzy-dependent pathway [63]. Capsules in serotype 3 and 37 are synthesized and exported using a synthase-dependent pathway. The gene loci in the 92 Wzy-dependent serotypes and serotype 3 are found between *dexB* and *aliA* genes on the chromosome [8, 64]. In the case of serotype 37, the only gene [47] responsible for polysaccharide synthase is located at a different position on the chromosome [65].

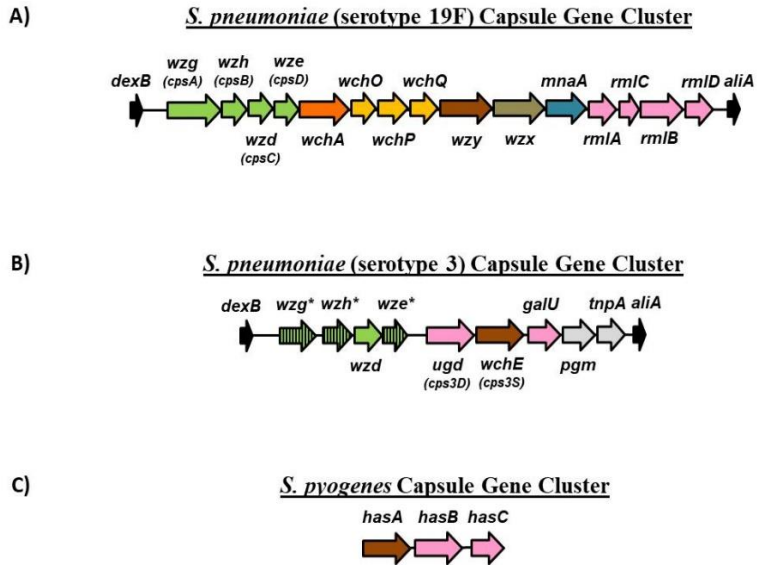


Figure 4. Organization of genes in capsular gene cluster of Gram-positive bacteria. A) Capsular gene cluster from *Streptococcus pneumoniae* (serotype 19F) is used as a model to describe the general architecture of genes in Gram-positive bacteria that use the Wzy-dependent pathway. Four conserved genes involved in modulation of capsule synthesis are colored in green, followed by initiating glycosyl transferase genes in orange, other glycosyltransferase and sugar modifier genes (*O*-acetylases) in yellow, wzy polymerase gene in brown, wzx flippase gene in tan and finally the genes for sugar nucleotide synthesis in pink. B) and C) are capsule gene clusters of Gram-positive bacteria using the synthase-dependent pathway. Genes encoding synthases are colored in brown. The conserved regulatory genes *S. pneumoniae* (serotype 3) are not transcribed (green with *) and are not essential for capsule biosynthesis.

In the Wzy-dependent pathway, *cpsABCD* (*wzg*, *wzh*, *wzd*, *wze*), genes are present on the 5' end of the gene locus (Figure 4A). These genes are highly conserved among all serotypes and are thought to regulate capsule synthesis. In more than 70% of serotypes the *cpsABCD* region is followed by a gene that encodes an initiating phospho-glycosyltransferase, WchA, that attaches the first sugar by transferring glucose-1-phosphate to undecaprenyl phosphate (C55 lipid). The remaining 30% of the serotypes use a different sugar to initiate polysaccharide synthesis. The rest of the gene locus in the 3' end encodes for enzymes that catalyze; polymerization (Wzy polymerase), transportation across membrane (Wzx flippase), glycosyl transfer (glycosyltransferases), sugar modification (*O*-acetylases), and synthesis of sugar nucleotides unique to the polysaccharide capsule. The whole gene locus in *S. pneumoniae* is transcribed as a single unit, starting at the primary promoter on the 5' end of *cpsA* [63].

While, the gene locus in *S. pneumoniae* serotype 3 (Figure 4B) contains the four conserved genes at the 5' end, these genes are not transcribed [66]. The transcription starts at a promoter

located after the conserved genes. Of the six transcribed genes on the 3' end Cps3D (UDP-Glc dehydrogenase) and Cps3S (synthase) are essential for polysaccharide synthesis.

3.2.2. Other Gram-Positive Bacteria

Streptococcus pyogenes or group A streptococcus (GAS) produces a hyaluronic acid (HA) capsule that is essential for its virulence. The HA capsule is made-up of alternating N-acetyl glucosamine and glucuronic acid residues [67]. GAS uses a Synthase-dependent pathway and its gene cluster (Figure 4C) "*hasABC*" encodes for three proteins, one of which (*hasC*, that encodes for UDP-glucose phosphorylase to synthesize UDP-glucose) is not essential for capsule synthesis. *hasA* and *hasB* genes encode for Hyaluronan synthase and UDP-glucose respectively and are the only two genes required for synthesis and export of capsule [68].

The presence of sialic acids on the side chains of all 9 serotypes of *S. agalactiae*, group B streptococcus (GBS) is a unique feature among all Gram-positive bacterial species and the arrangement of genes in the capsular gene locus reflect this [69]. GBS utilizes a Wzy-dependant pathway and its gene locus bears a close resemblance to that of *S. pneumoniae*. It starts with conserved *cpsABCD* genes that extend into a less conserved region encoding serotype specific proteins. In addition to this, the 3' end of the GBS gene locus contains 5 genes that encode proteins responsible for addition of sialic acid as the terminal sugar [69, 70].

Of the 13 *Staphylococcus aureus* serotypes identified, serotype 5 and 8 are the most common clinical isolates in humans [3]. The capsule in both of these serotypes contains three common sugar residues that differ only in their glycosidic linkages, and position of acetyl modifications [16]. To reflect this, the capsular gene cluster in *S. aureus* contains 16 genes *capABCDEFGHIJKLMNOP*, of which 12 are closely matched. Serospecificity between serotype 5 and 8 arises from *capHIJK* genes, that are flanked by 5' and 3' regions that are highly conserved [71]. *S. aureus* uses the Wzy-dependant pathway and out of 11 (among 16) essential genes in its capsule gene cluster, functions of only 5 gene products were determined.

4. CAPSULE BIOSYNTHESIS

The biosynthesis and assembly of capsular polysaccharide is a complex process. Though much progress has been made by a number of labs in understanding the mechanism involved, there is more to be discovered in order to paint a complete picture of the process. Here, we have described some of the current findings on the biosynthesis of capsular polysaccharides using the Wzy, synthase and ABC transporter pathways. For a more complete review of biosynthesis and assembly of capsular polysaccharides, please see reviews by Whitfield, Yother, DeAnglis, Howell and Zimmer [4, 72-76].

4.1. Wzy Dependent Pathway

The Wzy dependent pathway initiates capsule synthesis with the reversible transfer of a sugar 1-phosphate, commonly from a UDP- sugar to the C55 lipid, undecaprenol phosphate (Und-P) on the cytoplasmic face of the inner membrane [77]. In *E. coli* group 1 and 4 capsules this process is catalyzed by the initiating transferases (GTs); WbaP and WecA respectively [4]. WbaP and WecA are integral membrane proteins with the critical residues for catalysis located

in a large cytoplasmic domain. Subsequently the initiating molecule (Und-PP-sugar) is further modified by a series of monofunctional GTs to form the basic repeat unit of the capsular polysaccharide. The repeat units are built on the inner face of the cytoplasmic membrane and are subsequently transported to periplasmic space by Wzx flippase where it is elongated by the polymerase, Wzy [78-80]. Elongation of the polymer occurs in a block-wise manner. This elongating mechanism involves the transfer of the repeat units on the nascent polymer from its Und-PP unit to the new carrier lipid repeat unit at its reducing end (Figure 5). The capsular polysaccharide is then translocated to the surface of the cell. The precise mode of translocation to the cell surface is still being explored and could involve the use of multiple proteins [81]. However, the lipoprotein Wza has been identified as one of the essential components involved in this translocation process [82].

4.2. Synthase Dependent Pathway

The Synthase dependent pathway is responsible for the synthesis of capsular polysaccharide in both Gram-negative and Gram-positive bacteria. One of the capsular polysaccharide structures synthesized by this pathway is hyaluronan (HA). HA is a linear polysaccharide comprised of alternating glucuronic acid (GA) and *N*-acetylglucosamine (GlcNAc). Hyaluronan synthase is the enzyme responsible for the synthesis of HA [73, 83]. Capsular polysaccharides composed of HA can be found in both Gram-negative and positive bacteria. However, the mode of synthesis differs in each class of bacteria. Consequently, HAS responsible for synthesis is therefore varied and has been classified into two classes, class I and class II for Gram-positive and Gram-negative, respectively.

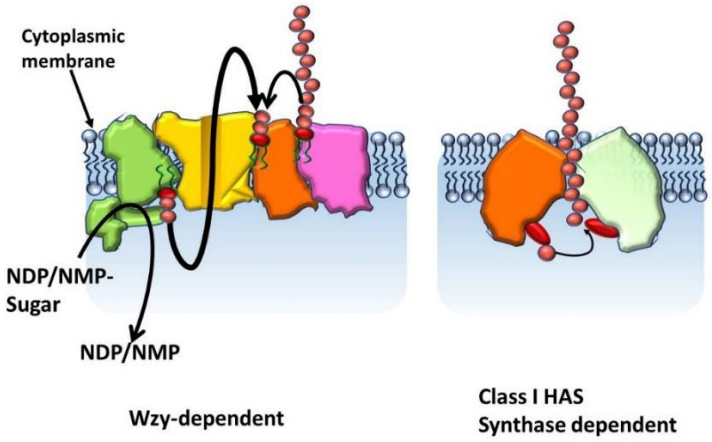


Figure 5. Models for the Wzy and Synthase dependent pathways for capsule synthesis and export. In the Wzy dependent pathway undecaprenyl diphosphate linked repeat units are synthesized by glycosyltransferases (depicted in green) on the cytoplasmic face of the membrane. It is then exported to the periplasm by the Wzx flippase (depicted in yellow). The undecaprenyl diphosphate linked repeat units are further elongated in a block-wise manner by the polymerase, Wzy (depicted in orange) which transfers the growing chain to the incoming repeat unit. In the Synthase dependent pathway, a single enzyme is responsible for synthesis and transport of the capsular polysaccharide. For class I hyaluronan synthase (HAS) the enzyme forms a homodimer with two functioning HAS domains. It is postulated that the dimer interface may serve as a transmembrane membrane channel for polysaccharide export to the cell surface.

To date the only identified HAS found in Gram-negative bacteria is from *Pasteurella multocida* Type A. *P. multocida* Type A HA capsule synthesis involves polymerization of HA bound to lipid-anchor in a non-processive manner by a membrane-associated hyaluronan synthase (*Pm*HAS) [84]. The Class II *Pm*HAS adds the monosaccharides sequentially with two separate GTase domains (one for the hexosamine and one for the uronic acid) [85, 86]. The synthesized polymer is then transported to the cell surface from the cytoplasmic face of the cell by an ABC transporter with the aid of periplasmic and outer membrane components [74].

However, in Gram-positive bacteria including *Streptococcus* and *Bacillus* a single enzyme is responsible for initiation, polymerization and transport of the growing polymer [72, 87]. These synthases are membrane integrated proteins and are members of the processive β-glycosyltransferases of the glycosyltransferase 2 (GT2) family. In addition, they contain four transmembrane [54] domains that frame the cytosolic GT domain. The ability for the hypothesized single enzyme to not only catalyze the addition of two different sugars but to also transport the synthesized polymer to the cell surface has been quite intriguing. Results from work in the Zimmer group has shown that the enzyme functions as a homodimer, where two functioning HAS domains are required for catalysis. In addition, it has been postulated that the dimer interface may serve as a TM channel that is energized by the polymerization reaction (Figure 5). It is unclear how Class I HAS or other such synthases are able to maintain the strict alternating sugar repeats. However, it has been proposed that the binding of the newly incorporated sugar alters the affinity of the site of the subsequent UDP-sugar [72, 87]. The mechanism of chain termination by the Class I HA synthases is still unknown.

4.3. ABC Transporter Dependent Pathway

E. coli group 2 capsules, as well as capsules found on *N. meningitidis* and *H. influenzae* are synthesized via the ATP-binding cassette (ABC) transporter dependent pathway. The biosynthesis of the capsular polysaccharide occurs on the cytoplasmic face of the inner membrane. In this pathway the assembled polysaccharides are exported from the cytoplasm during synthesis or after synthesis by the ABC transporters, KpsM and KpsT with the hydrolysis of ATP as an energy source. The temporal relationship of assembly and transport is yet unclear. A model put forth by the Vimr group [88] suggests that there is contact between the polysaccharide synthesis machinery and export components. If this model is correct it proposes that the polymerase catalytic efficiency is influenced by the operation or structure of the export components. This interaction could then serve as a feedback mechanism to regulate capsule synthesis.

The proteins, KpsE and KpsD (*E. coli* nomenclature) are highly conserved across *E. coli* group 2 capsule synthesis. KpsE and KpsS are essential for export and translocation. In addition, homologs of these proteins are also found in other bacteria that use this mechanism. It has been determined that *E. coli* and *N. meningitidis* capsules contain the same glycolipid at the reducing terminus of the CPS [89]. It consists of a lyso-phosphatidylglycerol moiety with a β-linked poly-KDO attached as linker to the reducing terminus of the CPS. This common feature of the CPS across multiple Gram-negative pathogens could serve as a conserved recognition molecule for the ABC transporter used to deliver CPS to the periplasm.

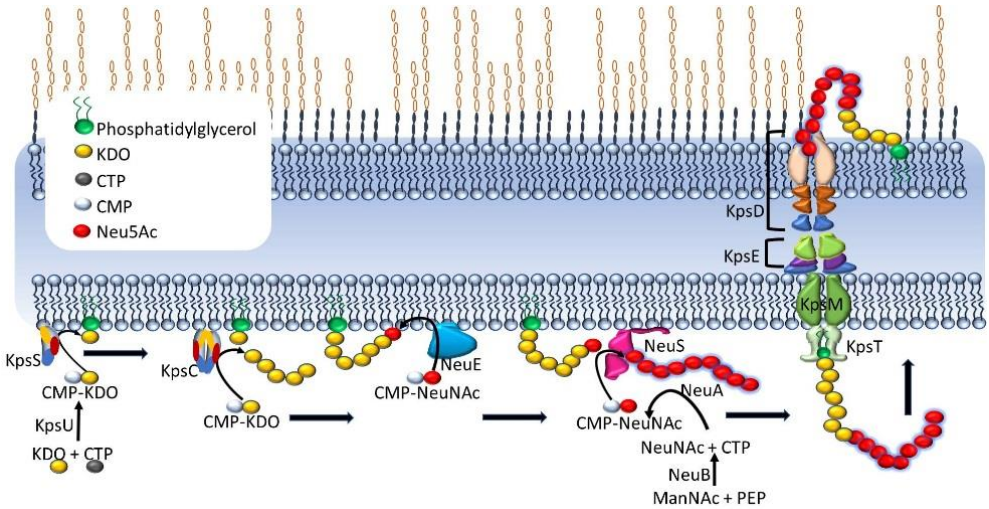


Figure 6. ABC transporter dependent synthesis of *E. coli* group 2 polysialic acid capsules.

The glycosyltransferases that synthesize the growing polysaccharide chains in the ABC transported dependent pathway are not self-initiating, and thus require the addition of an acceptor to observe transfer of sugar from sugar nucleotide precursor *in vitro* [90]. It has been proposed that this polyKDO containing glycolipid is the endogenous natural acceptor for initiating chain elongation. This model would imply that the glycosyltransferase that adds the first of the repeat unit to initiate synthesis would add directly to the polyKDO. It is presumed that in the biosynthesis of polysialic acid capsules the protein NeuE adds the first sialic acid residues to capsular polysaccharide forming the priming molecule for the polysialyltransferase (NeuS) (Figure 6). It should be noted that in K5 and other capsule synthesis there is no evidence of a NeuE homolog and therefore may not require a NeuE type protein to form a priming molecule. This scenario would suggest that the polymerase for capsules that don't contain polysialic acid proceed with synthesis by direct addition of the sugar repeat units to the poly-KDO lipid moiety.

Significant progress has been made on the synthesis of this putative initiation molecule. It has been determined that the highly conserved KpsS and KpsC proteins are essential for the synthesis of the glycolipid [89, 90]. Both KpsS and KpsC function as β-KDO transferases where KpsS adds the first KDO to the glycolipid and KpsC adds the additional KDO residues to the primer forming the poly-KDO linker [91]. KpsC has 2 KDO transferase domains that alternate in the addition of sugar in a β(2-4), β(2-7) fashion. Synthesis of polyKDO chains up to 20 units long *in vitro* were recently reported.

As to be expected there is considerable diversity among the enzymes that are involved in polymer elongation. Progress has been made in the study of the GTs responsible for polymer biosynthesis. In several cases the polymerizing enzyme catalyzes the formation of more than one linkage. In the case of the *N. meningitidis* serogroup W and Y the polymerizing enzymes have glucosyltransferase or galactosyltransferase domains linked to the sialyltransferase domain. Similarly, Litscho et al. reported that the polymerizing enzyme CslB of *N. meningitidis* has 2 domains and synthesizes a trisaccharide repeat 3)β-GlcNAc (1-3)β-GlcNAc (1-3)α-GlcNAc(1-OPO_3. Thus, this enzyme should have both glycosyltransferase and

phosphotransferase activities. The polysialyltransferases from *N. meningitidis* serogroup B and C as well as *E. coli* serotypes K1 and K92 have been identified [92-94]. A crystal structure has been reported for an α(2-8) polysialyltransferase from *Mannheimia haemolytica* serotype A2 [95]. This enzyme synthesizes a polysaccharide that is identical to *N. meningitidis* serogroup B and *E. coli* K1 and shares significant sequence homology. The *M. haemaolytica* enzyme has a GT-B fold with a polysialic acid binding groove formed by 2 Rossmann like domains.

Although, the K92 polysialic acid has both α(2-8) and α(2-9)NeuNAc linkages it does not appear that the linkages are catalyzed by separate domains. As suggested by Steenbergen et al. [94], the linkages are likely formed by reorientation of the acceptor or donor substrates in a single active site. A crystal structure would be very helpful in clarifying the reactions catalyzed by the K92 polysialyltransferase and the CslB transferase.

5. Technologies for Preparation of Glycoconjugate Vaccines and Therapeutics Based Capsular Polysaccharides

5.1. Current Technologies

Antibodies against the capsular polysaccharides of pathogenic bacteria are often protective against specific serogroups. However, since purified polysaccharides are not effective vaccines for humans with relatively immature immune systems and since they lack the ability to induce a boostable antibody response, glycoconjugate vaccines were developed. These glycoconjugate vaccines have been very successful at preventing diseases caused by *Neisseria meningitidis*, pneumococcus, *Haemophilus influenzae* type b, and *Salmonella typhi* in most populations and age groups. The success of these vaccines has been documented extensively in the literature and is evident from the growing number of licensed vaccines based on conjugated polysaccharides or oligosaccharides. These vaccines are prepared by the coupling of polysaccharides or large oligosaccharides derived from polysaccharides to a carrier protein, often a toxoid of diphtheria toxin or tetanus toxin. A list of the chemistries used to manufacture the vaccines currently licensed in the USA and Canada is given in Figure 7. All the methodologies described in this table have a degree of randomness and consequently, can result in a complex mixture of species. Most commonly, the saccharide antigen is activated by periodate oxidation to generate aldehydes and subsequently coupled by reductive amination to protein lysines or to hydrazides introduced onto the protein surface carboxyl groups. A variation on this approach employs an activation step at end of the saccharide chain with subsequent random single point attachment to lysine side chains on the protein. Thus, it is easy to see how complex mixtures would be generated requiring extensive analysis to characterize and insure process consistency. As a result, regulatory authorities and vaccine manufacturers have devoted much effort to this issue to insure consistency of glycoconjugate vaccines post licensure. The manufacture and analysis of glycoconjugate vaccines derived from bacterial polysaccharides has been reviewed elsewhere [96-98]. The problems associated with glycoconjugate manufactured by current technologies with a high degree of randomness can be reduced by development of more well defined glycoconjugates. In addition to relieving some

of the problems of manufacturing and analysis, better defined glycoconjugates would facilitate a more detailed interrogation of the immune system to determine the relevant structures for immunization.

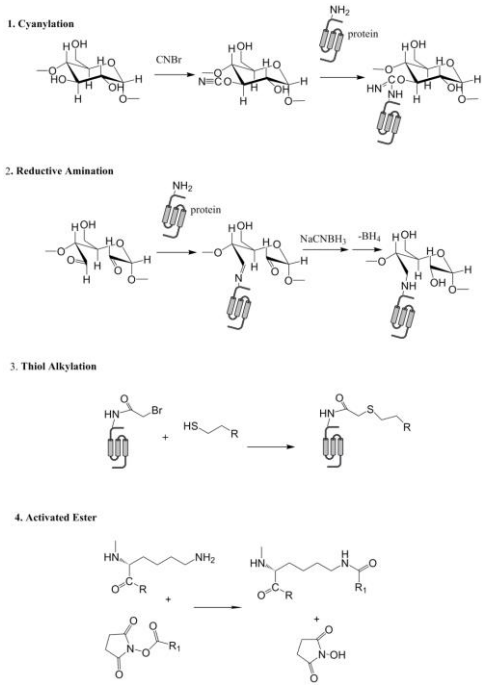
5.2. Chemical Synthesis of Glycoconjugate Vaccines

One approach to improving glycoconjugate manufacturing that is being tested is chemical synthesis of the saccharide antigen portion of the vaccines. A vaccine for *H. influenzae* type b is based on the complete chemical synthesis of oligosaccharide antigen. This vaccine was reported to be effective in humans and had increased control over the structure of the drug substance [99, 100]. Several vaccines have been prepared based on total chemical synthesis of O-antigen oligosaccharides of LPS and will not be discussed further. As mentioned, the Seeberger group has developed technology to define the immunologically relevant epitope of pneumococcal polysaccharides based on chemical synthesis of repeating oligosaccharides and variations on the oligosaccharide repeat units of the polysaccharide [9, 10, 101, 102]. Unlike relying on derivatives prepared from the natural polysaccharide, chemical synthesis allows complete control so that groups can be added or removed from the repeat unit to define the epitope. Glycoconjugates prepared in this fashion are first tested in arrays against pooled human reference serum for reactivity, then used to immunize mice to determine immunogenicity. The group has reported results on the repeats of several polysaccharides of *S. pneumoniae* in currently licensed vaccines [9, 10, 101, 102].

5.3. Emerging Technology Based on the Biosynthetic Toolbox

Recent developments in our understanding of how bacteria assemble extracellular polysaccharides has led to emerging technologies that promise greater specificity in conjugation and *in vivo* methods of production. The discovery of the bacterial oligosaccharyl transferase PglB in *Campylobacter* and the understanding of the similarities of polysaccharide assembly mechanisms with *N*-linked glycoprotein synthesis [103, 104] has presented an opportunity for assembly of glycoconjugate vaccines directly in *E. coli*. Some polysaccharides are assembled on an isoprenoid carrier at the reducing end and are subsequently transferred as an oligosaccharide to a glycolipid acceptor, i.e., LPS core. PglB is an oligosaccharide transferase that transfers the oligosaccharide for the isoprenoid carrier lipid to the asparagine of a protein in an analogous fashion [103, 104]. Thus, bacterial polysaccharides can be assembled as glycoconjugates in *E. coli* engineered with the necessary glycosyltransferases and carrier proteins containing the appropriate consensus sequence for PglB [105]. This technology has been primarily used as a proof of concept to attach the O-antigens of Gram-negative bacterial lipopolysaccharides to carrier proteins. Vaccines against *Shigella* prepared in this fashion with *Shigella* O-antigens have entered clinical trials [106]. However, recently, the biosynthetic enzymes of *S. aureus* capsular polysaccharides types 5 and 8 have been coupled to enzymes of the *Pseudomonas* O-antigen synthesis and PglB to produce glycoconjugates *in vivo* in *E. coli*. These glycoconjugate vaccines induced antibodies that are protective in mice [107].

Polysialic acid glycoconjugate structures have also been attached to proteins using the polysialyltransferases of capsular polysaccharide biosynthesis of *Neisseria meningitidis* group B. The Aebi group described a cytoplasmic *N*-glycosyltransferase (NGT) in *Actinobacillus pleuropneumoniae* that transfers glucose and galactose residues to an NX(S/T) consensus sequence [108]. Keys et al. engineered a biosynthetic pathway in *E. coli* using this *N*-glycosyltransferase along with sialyltransferases to polysialylate proteins [109]. The *Neisseria* polysialyltransferase requires a disialyl acceptor to begin polymer elongation. Keys first attached a glucose residue to the protein, then used the *Neisseria* galactosyltransferase (LgtB) and *Campylobacter* sialyltransferase (CstII) in sequence to generate a disialyllactosyl substrate for the NmB PST. The glycoprotein target was engineered with the consensus sequence of *A. pleuropneumoniae* NGT and expressed in *E. coli* harboring this pathway. The protein isolated from this construct was polysialylated at specific sites as expected with polysaccharides of lengths varying from 10 to > 80. These polysialic acids were sensitive to a α (2-8) specific endoneuraminidase and reactive to α (2-8) specific anti-polysialic acid.



Saccharide	Carrier Protein
Hib polysaccharide	tetanus toxoid
MenACYW polysaccharide	tetanus toxoid
Hib oligosaccharides	CRM197
MenA polysaccharide	tetanus toxoid
Pneumococcal polysaccharides	CRM197
MenACYW oligosaccharides	
Hib-polysaccharide	Meningococcal OMP
MenACYW oligosaccharide	CRM197

Figure 7. Common coupling chemistries for licensed glycoconjugate vaccines.

McCarthy et al. used a similar pathway *in vitro* to chemoenzymatically attach the α2-9 polysialic acid to tetanus toxin Hc fragment [110]. The disialic acid acceptor substrate was prepared on chemically synthesized lactosides of aliphatic azides by enzymatic sialylation with CstII. The products of the CstII reaction were polysialylated with the *N. meningitidis* group C PST yielding oligosaccharides that could readily be coupled to a carrier protein by "click" chemistry. The polysialylated products were randomly coupled to tetanus toxin Hc fragment

and used to vaccinate mice. Antibodies induced by these glycoconjugates were specific for NmC polysaccharide.

Recently Oldrini et al. combined the use of synthetic acceptor molecules bearing a conjugatable linker with enzymatic synthesis using biosynthetic enzymes to prepare glycoconjugate vaccine with a desired oligosaccharide size distribution [111]. The authors developed a construct of the CsxA polymerizing phosphotransferase that allowed chain length control based on knowledge of the mechanism of chain elongation. They coupled the enzyme to a solid phase and used a flow system to synthesize conjugatable oligosaccharides of the *N. meningitidis* group X polysaccharide of the desired size distribution. Mice immunized with glycoconjugates prepared from these oligosaccharides produced antibodies comparable to antibodies from mice immunized with glycoconjugate prepared with conventional protocols. Further development of such technologies promises to yield more efficient and well-defined methods for glycoconjugate vaccines for preventing disease.

Metabolic engineering as a tool for expression of glycosaminoglycans (GAG) based on bacterial polysaccharide biosynthesis has been recently reviewed by Williams, Linhardt, and Koffas [112, 113]. In addition to hyaluronic acid being produced as a capsular polysaccharide in Gram positive bacteria, chondroitin and heparosan are capsular polysaccharides of *E. coli* K4 and K5 respectively [23, 24]. These GAGs are group 2 capsules of *E. coli*. Metabolic engineering of heparosan, hyaluronan, and chondroitin is of interest because of a variety of biomedical applications of these polymers such as anticoagulation drugs, hydrogels, cosmetics and osteoarthritis treatment. Several strategies such as optimization of pathway regulatory elements, control of precursor production, and selection of pathway components has led to outcomes such as increased yields, changes in modification, and chain length. Due to the vulnerability of animal sourced heparin toward undesirable impurities, non-animal sources are being developed in the Linhardt and Liu labs [113, 114]. These investigators are using chemoenzymatic approaches to prepare biologically active heparin oligosaccharides. The enzymes derived from the bacterial pathways for synthesis of GAG-like capsular polysaccharides have been very helpful in this effort. A low molecular heparin oligosaccharide (12 mer) was produced at gram scale in a scheme involving enzymes from *P. multocida* and shown to have several heparin-like biological activities in animal models [114].

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