ANNUAL REVIEWS

Annual Review of Microbiology

Assembly of Bacterial Capsular Polysaccharides and Exopolysaccharides

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Annu. Rev. Microbiol. 2020. 74:521–43

First published as a Review in Advance on July 17, 2020

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-011420-075607

Keywords

extracellular polysaccharide, capsular polysaccharide, capsule, exopolysaccharide, glycan biosynthesis, glycan export

Abstract

Polysaccharides are dominant features of most bacterial surfaces and are displayed in different formats. Many bacteria produce abundant long-chain capsular polysaccharides, which can maintain a strong association and form a capsule structure enveloping the cell and/or take the form of exopolysaccharides that are mostly secreted into the immediate environment. These polymers afford the producing bacteria protection from a wide range of physical, chemical, and biological stresses, support biofilms, and play critical roles in interactions between bacteria and their immediate environments. Their biological and physical properties also drive a variety of industrial and biomedical applications. Despite the immense variation in capsular polysaccharide and exopolysaccharide structures, patterns are evident in strategies used for their assembly and export. This review describes recent advances in understanding those strategies, based on a wealth of biochemical investigations of select prototypes, supported by complementary insight from expanding structural biology initiatives. This provides a framework to identify and distinguish new systems emanating from genomic studies.

Contents

1. INTRODUCTION

Surface glycoconjugates (complex carbohydrate-containing macromolecules) are found in a variety of structural formats with varying distributions (105). Some are limited to particular phyla, such as lipopolysaccharide (LPS) glycolipids in gram-negative outer membranes, or wall teichoic acids (WTAs) and secondary wall polymers covalently linked to gram-positive peptidoglycan. Other glycoconjugate types span those boundaries. Examples are provided by glycosylated proteins, as well as capsular polysaccharides (CPSs) and secreted exopolysaccharides, which are the focus for this review.

CPSs are characteristically retained on the cell surface after centrifugation and form a surface-covering capsule structure revealed by microscopy. Although capsules have been reported in many species, covalent anchoring systems are currently limited to capsular glycolipids found in various gram-negative mucosal pathogens, or linkage to peptidoglycan in some gram-positive bacteria. At the other extreme, secreted exopolysaccharides lack robust surface association postsynthesis. In practice, the capsule/exopolysaccharide designations are often arbitrary, and most polymers fall somewhere between the two extremes, with glycan products distributed between cell-associated and secreted forms. In most cases, it is impossible to predict the extent of cell association based on the chemical structures of the polysaccharides, their cellular function, or the assembly pathways. Here, these glycans are collectively referred to as extracellular polysaccharides (EPSs).

Given the biological diversity of EPS producers, it is not surprising that there exists a remarkable catalog of known structures compiled in the Carbohydrate Structure Database (CSDB; **http://csdb.glycoscience.ru/database/**) (101). These are mostly assembled from identical repeat units of varying sizes and complexities, and relevant examples are given in the figures in this review. Polysaccharides are an excellent source of surface diversity due to variations in monosaccharide components, anomeric configuration, linkage positions, and the incorporation of noncarbohydrate substituents. For example, conventional serological typing has identified more than 90 different capsule serotypes in *Streptococcus pneumoniae* and almost 80 in *Klebsiella pneumoniae* (78, 114). New structures can be generated via mutation, gene duplication, or lateral gene transfer, suggesting the potential numbers are higher. Factors including host immune systems and immunization may promote diversity, but bacteriophages that use the capsule as a receptor likely provide the most prevalent source of selective pressure for structural diversification (70).

Despite the range of structures, EPS assembly and export follow one of four main assembly-export strategies. Three of these exploit nucleotide sugar-dependent glycosyltransferases (GTs) (45) for their synthesis and are distinguished by characteristic export machineries and the potential involvement of lipid-linked intermediates. The other is a solely extracellular process, requiring oligosaccharide or polysaccharide donor substrates. The goal of this review is to identify essential concepts using important prototypes, but space limitations preclude covering the many subtle variations found in representatives of each assembly strategy. These assembly-export systems are employed in different contexts in bacteria with different lifestyles, so most are subject to complex (frequently species-specific) transcriptional regulation, and this important element is also beyond the scope of this review.

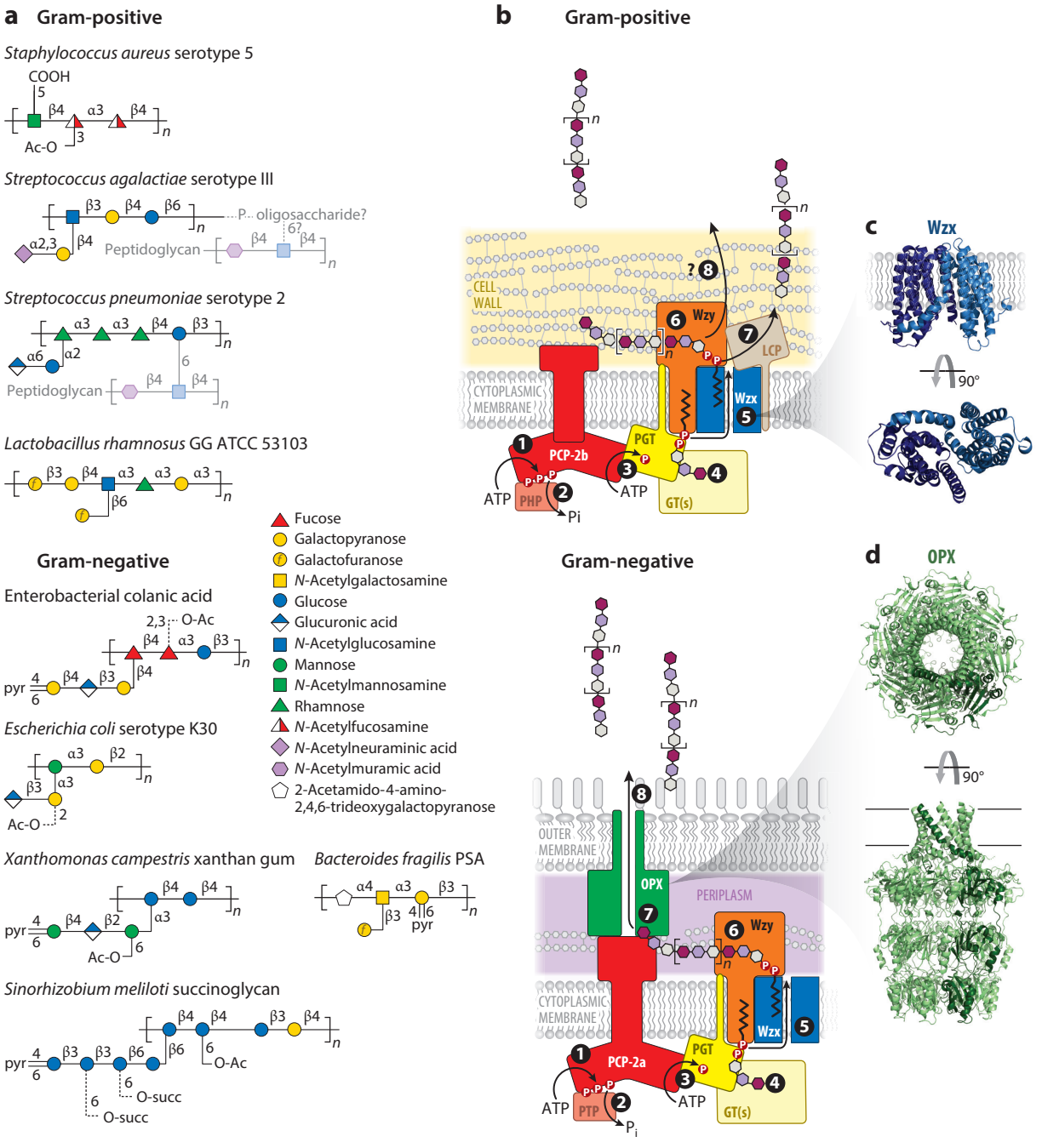
2. Wzy-DEPENDENT ASSEMBLY OF COMPLEX BRANCHED HETEROPOLYSACCHARIDES

The most widespread strategy for EPS production is known as the Wzy-dependent pathway, and it is found in both gram-positive and -negative bacteria (**Figure 1*a***). The foundation of this system was first described in LPS O-antigen biosynthesis and named after the putative polymerase, Wzy (formerly Rfc). Given conserved Wzy membrane topology, sequence motifs, mutant phenotypes, and assumed function, the designation is now broadly applied to polysaccharide biosynthesis in different genera. This system is used to build many capsules, which protect pathogens against host innate immune defenses by suppressing inflammatory responses and providing resistance to antimicrobial immune peptides, complement-mediated killing, and phagocytosis. For example, capsules are vital virulence determinants in *S. pneumoniae* (reviewed in 78). However, anti-CPS antibodies may protect the host against infection by some encapsulated bacteria (including pneumococci), and this has driven the development of successful multivalent CPS:protein conjugate vaccines (66). Some Wzy-dependent EPSs can have beneficial effects for the host. For example, a single isolate of the commensal *Bacteroides fragilis* may produce as many as eight phase-variable capsule types, and the zwitterionic PSA (polysaccharide A) is important in T cell activation and homeostasis of the immune system (32). A variety of immunomodulatory and health-promoting activities have also been attributed to other Wzy-dependent EPSs produced by lactic acid bacteria, offering new opportunities in functional foods (121).

Many plant-associated bacteria use this method to assemble EPSs. *Sinorhizobium meliloti* succinoglycan EPS is involved in the formation of nitrogen-fixing nodules in legumes, and the stoichiometry and positions of *O*-succinyl modifications impact activity and host specificity (61, 65). In plant pathogens causing vascular diseases (e.g., *Xanthomonas campestris* and *Erwinia* species), EPSs are crucial virulence determinants, although their precise roles are not always fully

understood. Like those from human pathogens, some can suppress signaling events in eliciting immune responses (3) and facilitate evasion of plant immune defenses (19).

Some Wzy-dependent EPSs participate in biofilms. In *Pseudomonas aeruginosa*, Psl (polysaccharide synthesis locus) EPS shares overlapping roles with Pel (pellicle formation) EPS (a synthase



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Wzy-dependent pathway. (*a*) Examples of repeat-unit structures of EPSs assembled by this strategy and proposed linkage to peptidoglycan in some gram-positive bacteria. (*b*) Hypothetical models for assembly and export in gram-positive and gram-negative bacteria. Steps ❶–❻ are conserved in both. (❶, ❷) Autophosphorylation/dephosphorylation of the PCP-2a/b proteins is required for EPS production. (❸) PGT (and other enzymes) may be activated by transphosphorylation. (❹) The Und-PP-linked repeat unit is synthesized by PGT and other assembly enzymes. (❺) Export via Wzx. (❻) Blockwise polymerization of Und-PP-repeat units by Wzy. In gram-positive bacteria, nascent EPS is transferred to peptidoglycan assembly intermediates and linked to the cell wall by LCP activity (❼) or potentially released by an unknown mechanism (❽). In gram-negative bacteria, the nascent polysaccharide is released by an unknown mechanism into a translocation pathway provided by the OPX and PCP-2a proteins (❼). Posttranslocation, the gram-negative EPS remains associated with the cell surface or is secreted into the environment. (*c*) Homology model of the structures of Wzx (*Escherichia coli* K30) in the inward-open conformation, based on MurJ (PDB 5T77) (the two six-helical bundles are colored in different shades of *blue*) and (*d*) the solved structure of the *E. coli* K30 OPX (Wza) octamer (PDB 2J58). Abbreviations: Ac, acetyl; EPS, extracellular polysaccharide; GT, glycosyltransferase; LCP, LytR-CpsA-Psr; OPX, outer membrane polysaccharide; PCP, polysaccharide copolymerase; PDB, Protein Data Bank; PGT, phosphoglycosyltransferase; PHP, polymerase and histidinol phosphatase; PSA, polysaccharide A; PTP, phosphotyrosine phosphatase; pyr, pyruvate.

product; see below) and contributes to pathogenesis (50). Colanic acid from *E. coli* participates in biofilm formation but may also confer the ability to survive conditions of envelope stress (reviewed in 103).

EPSs formed by this mechanism include some of the largest glycopolymers (molecular weight $>10^6$ Da) and may possess useful viscosity and pseudoplastic properties of commercial value. For example, lactic acid EPSs contribute to the texture and viscosity of fermented foods (117), while *X. campestris* xanthan gum is a particularly important biopolymer, with realized and proposed applications as a thickener for foods, cosmetics, paints, and tertiary oil recovery (95). Modified xanthan has additional utility in biomedical applications including drug delivery and in formulations of scaffolds and hydrogels for tissue regeneration (44).

In Wzy-dependent glycan biosynthesis (**Figure 1*b***), repeat units are assembled using cytosolic nucleotide sugar substrates and undecaprenyl phosphate (Und-P) lipid carrier embedded in the membrane bilayer. Synthesis of Und-PP-repeat units is initiated by a polyprenol phosphate phosphoglycosyltransferase (PGT) and completed by conventional Leloir GTs (45). Und-PP-repeat units are transported to the external face of the membrane by a transporter belonging to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) family (often designated Wzx), where Und-PP-linked glycan is then elongated by addition of repeat units by the pathway-defining Wzy polymerase. The same approach is used in biosynthesis of some LPS O antigens, where the Wzx and Wzy designations originated (reviewed in 36, 81, 106a), and comparable early reactions are involved in protein glycosylation pathways (30) and peptidoglycan biosynthesis (86). EPS applications of the Wzy-dependent strategy are distinguished by a particular class of polysaccharide copolymerase (PCP) protein that regulates polymerization and by proteins that determine the final cellular fate of the product.

2.1. Biosynthesis and Export of Extracellular Polysaccharide Repeat Units

Bacteria and eukaryotes using PGT enzymes initiate glycans in systems that use polyprenol-diphosphate linked intermediates. Bacterial PGTs belong in one of two families based on topology of the essential catalytic core and mechanism (reviewed in 2). Polytopic proteins include MraY (from peptidoglycan biosynthesis) and WecA (O antigen), while monotopic PGTs are represented by PglC (N-glycosylation) and WbaP (O antigen and EPS biosynthesis). While some of the monotopic group possess multiple transmembrane helices (TMHs), the essential catalytic unit is composed of the GT domain and a single TMH, in a format resembling PglC. The PglC active site sits in close proximity to the membrane surface, enabling catalysis with no requirement to displace the

Und-P acceptor from the bilayer (83). EPS PGTs generate Und-PP-linked hexoses (e.g., CpsE in some *S. pneumoniae* capsule serotypes and WbaP or WcaJ in *E. coli* and *K. pneumoniae* capsules and colanic acid), or *N*-acetylhexosamine residues (e.g., CapM from *S. aureus* serotype 5). The reaction products provide an acceptor for sequential assembly of the repeat unit by structure-specific GTs, and enzymes that add nonglycose components.

Und-PP-linked EPS repeat units are exported by MOP flippases, where peptidoglycan lipid II flippase (MurJ) offers the prototype. MurJ is composed of two 6-TMH bundles plus two additional TMHs, 13 and 14, and was proposed for transition between inward- and outward-open states (43, 120). In the inward-open conformation, the lipid part of the substrate is thought to bind in a hydrophobic groove along TMHs 13 and 14 [which are missing in prototypical EPS Wzx proteins (**Figure 1*c***)] and leads to a lateral portal between TMHs 1 and 8. The portal allows the glycan to access the positively charged lumen for export. The outward-open structure closes the cytosolic gate, and dynamic changes in the channel size are thought to facilitate release of substrate at the exterior face. How the export process is energized (e.g., possible involvement of counterions) and how the lipid is reoriented throughout the export cycle remain to be established. Wzx appears to use a similar mechanism, based on the ability of relaxed substrate-specificity mutants of Wzx from colanic acid biosynthesis to functionally replace MurJ (92). However, studies with O antigen Wzx proteins have illustrated complex substrate preferences for optimal activity (35) that likely applies to the EPS flippases.

2.2. Polymerization of Repeat Units

The proposed Wzy reaction transfers a growing glycan from its Und-PP carrier to the nonreducing terminus of the incoming Und-PP-repeat unit, although direct biochemical evidence is confined to an O antigen Wzy from *E. coli* O86 (112). Wzy polymerase topology shows ~12 TMHs and two large periplasmic domains with conserved motifs implicated in a speculative catalysis model (36). The periplasmic Wzy_C motif is shared with other enzymes catalyzing reactions involving Und-PP-oligosaccharide donors, including O antigen ligases and some oligosaccharyltransferases for O-linked protein glycosylation (91). Unfortunately, no Wzy structure is currently available to illuminate the mechanism, and the challenge is highlighted by resolution of the structure of bacterial oligosaccharyltransferase (PglB), which performs a comparable glycosylation mechanism at the membrane interface. Resolution of the structure and its dynamics was only achieved through elegant studies with enzyme-substrate complexes (71).

Polymerization requires a continuous supply of Und-PP from a limited pool that must also sustain peptidoglycan biosynthesis for growth. It is assumed that the cell must prioritize peptidoglycan because of its essential nature, but the processes that establish priority are unknown. Nevertheless, the importance of recycling spent Und-PP from the exterior face of the membrane to regenerate Und-P in the cytoplasm is highlighted by the morphological defects accruing in *E. coli* when the pool is diminished by defects in other nonessential pathways that sequester Und-P the pool (38). Although the process for retrograde transport of Und-P is unknown, overlapping periplasmic enzymes and processes facilitate Und-PP dephosphorylation in *E. coli*, and homologs (of some) exist in gram-positive bacteria (40). Direct coupling of one phosphatase to *E. coli* PBP1B peptidoglycan synthase alleviates product inhibition and illustrates the potential for close connections between the reactions (33).

2.3. Controlling Polymerization—The Polysaccharide Copolymerase Proteins

E. coli O86 Wzy is a distributive (as opposed to processive) enzyme, where product is released after each reaction and product abundance decreases with chain length (119), and it is reasonably

assumed that other Wzy homologs function in the same way. Generation of the observed EPS size and chain-length distribution therefore requires a process promoting chain extension, and this is performed by a PCP protein (99). PCP proteins possess a predominantly α-helical periplasmic domain flanked by two TMHs and oligomerize in a structure extending from the membrane. The majority of Wzy-dependent EPS systems are distinguished by PCP-2 proteins, which possess an additional cytosolic tyrosine P loop (BY) kinase absent in PCP-1 proteins from O antigen biosynthesis (reviewed in 17, 67). Tyrosine phosphorylation of the PCP-2 component, and other proteins in the biosynthesis pathway, plays an essential role in the ability to polymerize EPS. In most gram-negative examples, the transmembrane and C-terminal kinase domains are fused in a single PCP-2a protein; the prototype (Wzc) is from *E. coli* serotype K30 group 1 capsule and colanic acid biosynthesis. Gram-positive bacteria (and a few gram-negative bacteria), possess PCP-2b proteins where the two domains are separate polypeptides that are both required for EPS production; the prototypes are CpsBC in *S. pneumoniae* and CapAB in *S. aureus*. The structure of a CapAB chimera structure (comprising the CapB kinase plus a small C-terminal fragment of transmembrane CapA) revealed the penultimate residue in CapA stabilizes nucleotide binding in the kinase active site (76). Kinase domains in PCP-2a and PCP-2b proteins share the ability to oligomerize in octameric rings and catalyze intermolecular autophosphorylation of a tyrosine-enriched C terminus of an adjacent monomer (5, 76). An early low-resolution cryo–electron microscopy (cryo-EM) structure of full-length Wzc from *E. coli* showed a tetrameric organization with extensive contacts within the periplasmic domain and spatially separated kinase domains (14). The phosphorylation status of the tetrameric single particles is unknown, but it is currently believed that phosphorylation leads to dissociation of the kinase rings.

EPS production is influenced by the amount of PCP-2a/b phosphorylation, and BY-kinase activities are antagonized by cognate phosphatases, belonging to different families in gram-negative or -positive bacteria. Wzb in *E. coli* is a phosphotyrosine phosphatase representative, while CpsD in *S. pneumoniae* is a member of the polymerase and histidinol phosphatase family (29). Wzb docks near the Wzc active site (97). Genes encoding the PCP-2a/b and phosphatase proteins typically occur in a conserved block at the 5′ end of the EPS operons in different serotypes of a species, indicating functions independent of precise repeat-unit structures. However, some species possess additional unlinked BY-kinase/phosphatase genes, and these can provide overlapping functions (82, 113).

How does phosphorylation of PCP-2*x* proteins influence EPS production? One mechanism certainly involves modulating activities of other EPS biosynthesis proteins (reviewed in 67), and an elegant recent study with *S. aureus* serotype 5 provides detailed biochemical insight (82). Purified PGT (CapM), as well as an enzyme (CapE) required for UDP-FucNAc donor synthesis, were both substrates for in vitro phosphorylation by CapAB chimera. Mapping the phosphorylated tyrosine in CapM onto the PglC structure suggests the observed enzyme activation is due to protein stabilization. Tyrosine phosphorylation has been reported to be required for activity of the PGT enzyme (WcaJ) in *K. pneumoniae* (51), but the mechanism is unclear since the modified residue is located outside the catalytic core. Phosphorylation (and EPS synthesis) is anticipated to be influenced by transduction of (unknown) signals by the periplasmic PCP-2a/b domain (67), although additional regulatory factors may also come into play. For example, in *S. aureus*, the Ser-Thr kinase PknB phosphorylates CapB, downregulating the autokinase and opposing the BY-kinase activation of CapM (82). In *S. pneumoniae*, capsule production is integrated with the cell cycle. The BY-kinase is positioned at the mid-cell by interacting with ParB (the chromosomal segregation protein), and recruits biosynthesis proteins to position glycan production at the septum (75). Whether comparable processes occur in gram-negative systems is unknown, but given the different environments and stresses encountered by EPS producers, one might

anticipate varied additional layers and approaches to regulation in different bacteria. For example, the existence of a corresponding kinase domain for the PCP-2a-like protein is uncertain in the prodigious EPS producer *X. campestris* (17).

2.4. Terminal Steps in Gram-Positive Bacteria—Ligation to Peptidoglycan or Release?

Postpolymerization steps diverge in gram-positive and -negative bacteria. In gram-positive bacteria, attachment of EPS to peptidoglycan seems common. This is catalyzed by enzymes belonging to the LytR-CpsA-Psr (LCP) family, which also transfer WTA and other anionic secondary cell wall polymers to peptidoglycan (27, 39). In *S. pneumoniae* the conserved 5′ block of genes also encodes the LCP homolog (CpsA) in addition to the BY-kinase/phosphatase pair. Although CpsA is the primary capsule ligase in *S. pneumoniae* serotype 2, LytR and Psr can also contribute (24). In contrast, *S. aureus* possesses multiple *lcp* genes (12), which are not linked to the capsule locus, and LcpC is primarily responsible for capsule attachment (82), while LcpA is the major WTA ligase (88). CpsA binds polyisoprenoid phosphate lipids in a hydrophobic pocket whose properties are conserved in other LCP proteins, and two Asp residues in the catalytic site coordinate an essential Mg^{2+} ion that orients the Und-PP phosphates for hydrolysis of the pyrophosphate phosphodiester bond (39). The complete pathway for *S. aureus* EPS ligation by LcpC has been recapitulated in vitro, demonstrating transfer of Und-PP-linked intermediates to lipid II and subsequent polymerization of the product by peptidoglycan synthase PBP2 (82). Notably, LCP activity was enhanced by the presence of CapA, providing an additional role for the transmembrane partner of the BY-kinase, while PknB sensed lipid II levels to integrate capsule and cell wall biogenesis. The demonstrated phosphodiesterase activities of the LCP homologs raise questions about the chemistry of the linkage between CPSs. This established activity is partially consistent with reported CpsA-mediated formation of phosphodiester linkages in *Streptococcus agalactiae* group III EPS (100) (**Figure 1**), although the origin of the proposed oligosaccharide linker is unclear. Group III EPS is reported to be linked to *N*-acetylglucosamine (GlcNAc) residues, while WTA LCPs add glycans to *N*-acetylmuramic acid (MurNAc). A phosphodiester linkage is also proposed for *S. aureus*, but definitive structural information is unavailable (13). However, the direct glycosidic bond proposed in GlcNAc-linked *S. pneumoniae* capsules (46) (**Figure 1*a***) is difficult to rationalize with phosphodiesterase activity for the CpsA LCP protein. These apparently contradictory observations require reconciliation.

What of systems, like lactobacilli, that release substantial amounts of polysaccharide into the environment? Lactobacillus LCPs encoded by the EPS loci have been implicated in transcriptional regulation of the EPS-production genes, but definitive experimental evidence is limited (reviewed in 117). The sequence conservation suggests these LCP homologs catalyze peptidoglycan ligation, and copious EPS producers like *Lactobacillus rhamnosus* GG do generate a cell wall–associated capsular layer (49), although the linkage chemistry has not been established. In these bacteria, cell-free EPS may result from an abortive Wzy polymerization step or release by LCP without completion of ligation. In the absence of LCP function, some bacteria that normally link capsule to peptidoglycan do release secreted glycan (see 82). Alternatively, release could result from cell wall turnover during growth. Clearly there is more to learn.

2.5. Wza, the Outer Membrane Translocon in Gram-Negative Bacteria

The outer membrane size-exclusion barrier must be bypassed for translocation of EPS to the cell surface or into the external environment, and this is achieved by outer membrane polysaccharide (OPX) proteins (17), with *E. coli* Wza as the prototype. Wza is a lipoprotein that forms a stable

octamer (**Figure 1*c***) where the outer membrane barrel (internal diameter 17 Å) is composed of amphipathic α helices rather than the usual β-barrel outer membrane proteins (21). The majority of the octamer encloses a periplasmic lumen with a size sufficient to accommodate the radius of an EPS chain. The interior contains abundant polar residues and is conserved within a species, and these proteins likely operate independent of a specific EPS repeat-unit structure. Site-specific cross linking provided direct evidence for the transit of polymers through the Wza translocon (73) but offered no insight into how the substrate entered the lumen, which is closed at the periplasmic face by a ring of Tyr residues. One possibility is provided by a low-resolution cryo-EM structure showing the periplasmic domains of Wza and the Wzc BY-kinase interact, causing changes in the Wza base (15). Notably, deletion of *wza* downregulates polymer production (72), in a feedback process that presumably operates via the Wzc BY-kinase.

The current model assumes that the growing glycan may remain linked to Und-PP during chain extension and (potentially) translocation, but what happens to polymers emerging from the translocon? No dedicated enzyme has been identified to catalyze release of exopolysaccharides in these systems, and by analogy to the proposal for gram-positive systems, an abortive Wzy reaction could be involved. There is also no evidence for a covalent attachment of the glycan products to the cell surface, yet many examples (like the *E. coli* prototype and its *K. pneumoniae* counterparts) produce a capsule structure. The *E. coli* and *K. pneumoniae* genetic loci encode an outer membrane protein (Wzi) with lectin-like properties that plays a role in organizing the capsule structure (10). *wzi* mutants lose the coherent capsule structure and release more CPS. However, the Wzi protein is not widely disseminated among gram-negative capsule producers, so additional processes must be involved. Ionic associations with LPS have also been proposed to stabilize *K. pneumoniae* capsules (26), but neither approach convincingly explains the observed robust cell association. The number and distribution of active assembly complexes in the cell envelope remain to be determined.

3. THE ABC TRANSPORTER–DEPENDENT PATHWAY FOR GRAM-NEGATIVE CAPSULAR GLYCOLIPIDS

ATP-binding cassette (ABC) transporters comprise a large family of importers and exporters spanning the tree of life. Among their many functions, some ABC transporters export Und-PP-linked glycans for protein N-glycosylation and the biosynthesis of WTAs and LPS O antigens, while others export an important class of capsular glycolipids assembled in Und-independent processes in gram-negative bacteria (52). Most CPS examples are found in mucosal pathogens, where the resulting capsules are required for virulence. The same lipid and assembly strategy used in extraintestinal pathogenic *E. coli* (ExPEC) (e.g., serotypes K1 and K5), *Neisseria meningitidis*, *Haemophilus influenzae*, *Campylobacter jejuni*, *Actinobacillus pleuropneumoniae*, and several other pathogens of humans and livestock (22) (**Figure 2*a***). A variation of the pathway (with a different terminal lipid) is found in a Vi-antigen capsule produced by *Salmonella enterica* serovar Typhi and by some soil bacteria belonging to *Burkholderiales* (53). Effective capsule-based vaccines have been developed for *S.* Typhi and some serotypes of *N. meningitidis* and *H. influenzae* (reviewed in 66), but other targets are limited by molecular mimicry of host glycans. For example, *E. coli* K1 and *N. meningitidis* B possess polysialic acid capsules with the same structure as glycan chains on NCAM (neural cell adhesion molecule), while those of *E. coli* K4 and K5 resemble chondroitin and heparosan, respectively (16).

This biosynthesis strategy (**Figure 1*b***) is characterized by completion of the glycolipid in the cytoplasm, followed by export and translocation of the completed glycolipid to the cell surface via an ABC transporter coupled to cognate PCP-3 and OPX proteins. The prototypes are *E. coli* K1 and K5 group 2 capsules (104).

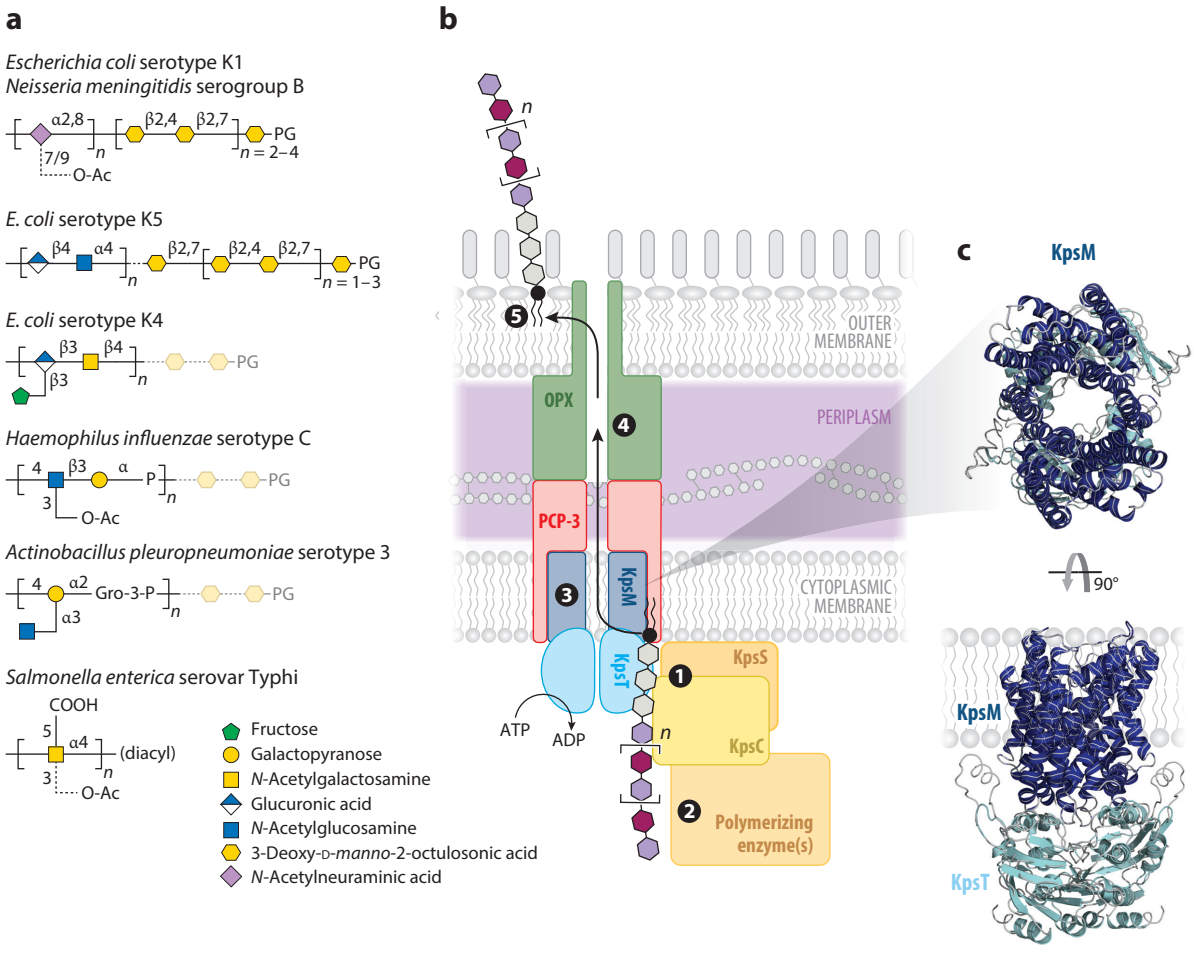


Figure 2

ABC transporter-dependent pathway. (*a*) Examples of repeat-unit structures and glycolipid termini of extracellular polysaccharides assembled by this strategy. (*b*) Hypothetical model for assembly based on the predominant representatives. (❶) Formation of the conserved glycolipid terminus using phosphatidylglycerol as the acceptor. (❷) Sequential transfer of components to complete the glycan chain. (❸) Export via the ABC transporter (KpsMT). (❹) Translocation in a pathway provided by the PCP-3 and OPX proteins. (❺) Insertion into the outer leaflet of the outer membrane. (*c*) Homology model of the ABC transporter structure (*Escherichia coli* K1 KpsM-KpsT) based on the O antigen transporter (Wzm-Wzt) from *Aquifex aeolicus* (PDB 6AN7). Abbreviations: ABC, ATP-binding cassette; Ac, acetyl; OPX, outer membrane polysaccharide; PCP, polysaccharide copolymerase; PDB, Protein Data Bank; PG, phosphatidylglycerol.

3.1. Cytosolic Polymerization

Almost all examples are built on the same phosphatidylglycerol acceptor. Although purified natural products mostly contain lyso-phosphatidylglycerol (109), this may be an artifact introduced during isolation and analysis. The purified GTs can use monoacyl and diacyl acceptors, and the low abundance and membrane-disrupting properties of lysophospholipids suggest phosphatidylglycerol is the authentic acceptor (22). A linker oligosaccharide of β-linked 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) is built on phosphatidylglycerol by two CMP-Kdo-dependent GTs. KpsS adds a single Kdo, while KpsC possesses two catalytic sites that then add

several alternating β2,7- and β2,4-linked residues (22). The β-Kdo oligosaccharide provides an acceptor for different repeat-unit structures, and the corresponding nucleotide sugar-dependent polymerases include single-site enzymes like polysialyltransferases (e.g., NeuS in *E. coli* K1) or multiple GT catalytic domains contributed by one (K4) or more (K5) polypeptides. Some examples combine GTs with hexose/hexNAc-1-P transferases (e.g., *H. influenzae* C) or glycerol-3-P transferases (*A. pleuropneumoniae* 3) in modular proteins resembling polymerases from WTA biosynthesis (55). In each system, a transition GT is presumably required to modify the β-Kdo oligosaccharide to create a suitable substrate for the polymerase. Although transition GTs are not yet experimentally assigned (110), their acceptor specificities (terminal β2,7- or β2,4-linked Kdo) may explain why the oligosaccharide linker in the final product can involve odd (e.g., *E. coli* K1) or even (K5) numbers of residues (109). The Kdo-linker bridging a lipid terminus to a variable glycan backbone is reminiscent of LPS, where all examples possess one or more Kdo residues. However, Kdo residues in LPS are α linked, and the corresponding enzyme (WaaA belonging to the GT30 glycosyltransferase family (90) is structurally distinct from KpsC enzymes belonging to the GT107 family, making KpsC a potential antivirulence therapeutic target (22).

Currently, only one variant of this biosynthetic model is known: the Vi-antigen EPS produced by *S.* Typhi and some *Burkholderiales*. These organisms lack the capacity to synthesize the β-Kdo oligosaccharide, and the Vi EPS possesses a reducing terminal diacyl-HexNAc moiety (54) (**Figure 2*a***). A Vi-specific acyltransferase (VexE) belonging to the lysophospholipid acyltransferase (LPLAT) family is required for proper acylation, but the source of the second acyl chain is unknown (54). The precise structure of the lipid terminus and the biosynthetic sequence for Vi-antigen EPS (i.e., whether acylation occurs prior to or after polymerization) are undetermined.

3.2. The ABC Transporter

Capsule transporters are composed of two transmembrane domains (TMDs) (designated KpsM in *E. coli*) and two nucleotide-binding domains (NBDs) (KpsT) (52) (**Figure 2*c***). The ABC transporter is conserved within a species and transferable between capsule serotypes (and occasionally between species), indicating that they operate without specificity for the repeat-unit structure (110). Presumably, the conserved terminal glycolipid is a critical element in substrate recognition; consistent with this, the acylation defect in an *S.* Typhi *vexE* mutant results in impaired export (54).

No capsule ABC transporter structure is currently available, but the Wzm-Wzt (Und-PP-O antigen) exporter offers insight into anticipated features (6, 11) (**Figure 2*c***). Unlike classical alternating-access transporters, Wzm TMDs form a contiguous membrane-spanning channel lined with aromatic residues to permit CH-π stacking interactions with the glycan substrate. The channel width is sufficient to accommodate a linear glycan chain but could potentially become challenging at its narrowest point for polymers with elaborate side chains; this might explain the absence of such side chains in glycans exported by this type of export system. Wzt NBDs possess a characteristic gate α helix positioned at the membrane-cytosol interface, which packs against the adjacent TMD protein and the opposite NBD to create a positively charged pocket, which is proposed to recruit the Und-PP-linked substrate. The glycan is proposed to enter the channel lumen with the lipid portion remaining in the membrane through the export cycle. Export is thought to be driven by rigid-body motion of the NBDs (dependent on the nucleotide-binding state) and conformational rearrangements that drive the gate loop and its interacting elements toward the periplasm, exerting a push on the substrate. An iterative cycle would export the glycan a few residues at a time. In this model the exporter behaves as a translocase (like synthase

enzymes; see below) rather than a conventional flippase. The KpsM and Wzm homology suggests a shared architecture, but the gate helix of Wzt is not apparent in KpsT (**Figure 2*c***), indicating different approaches to substrate engagement and translocation. For capsule transporters, the phosphatidylglycerol lipid must be removed from the membrane to complete translocation to the cell surface, and a solved structure is required to illuminate the process.

E. coli K1 *kpsT* mutants accumulate polysaccharides with larger average sizes than the wild type at the cytoplasm-membrane interface (47, 79), suggesting chain-length distribution is established by coupling of synthesis and export. A heterocomplex containing biosynthesis and export machinery has been identified in *E. coli* K5 (64), and loss (by mutation) of protein-protein interactions may release constraints on the polymerase.

3.3. Translocation to the Cell Surface

Like the Wzy-dependent pathway, translocation of the final product to the cell surface requires PCP and OPX proteins. However, the PCP proteins belong to PCP-3 (17), which lack BY-kinase domains. Cryo-EM images from reconstituted proteoliposomes containing the PCP-3 homolog from *N. meningitidis* B (CtrB) reveal a hexameric cone-like structure (resembling PCP-1 proteins) extending ~100 Å from the membrane and offering potential interaction with the corresponding OPX (47). Unlike OPX proteins from Wzy-dependent mechanisms, those affiliated with ABC transporter–dependent systems are not a homogeneous group. Many (e.g., *N. meningitidis* CtrA and *S.* Typhi VexD) are lipoproteins with predicted secondary structures resembling Wza, although VexD does not form sodium-dodecyl sulfate–stable multimers like Wza (87). In contrast, the *E. coli* OPX protein (KpsD) has a different predicted secondary structure, is not acylated, and lacks robust association with the outer membrane. Its translocation function requires an association with peptidoglycan mediated by murein lipoprotein (20). Differences in the structures of OPX proteins from the two assembly systems may have functional significance because delivery of a glycolipid into the outer membrane (as opposed to release at the cell surface) presumably requires some form of lateral gating in the transmembrane channel. Structures of these OPX proteins are now needed to direct further investigation. Regardless of variations in OPX structure, cognate PCP-3/OPX combinations seem to interface with the ABC transporters in the same way, based on functional cross complementation between *E. coli* and *N. meningitidis* (47). However, a single PCP or OPX mutant can only be complemented by expressing a cognate PCP-3/OPX pair, consistent with unique PCP-3–OPX interactions.

The current working model invokes a contiguous export-translocation pathway bridging the periplasm and outer membrane where glycolipid substrate passes from the ABC transporter into the PCP-OPX, resembling type I protein secretion (34). However, this may be an oversimplification because recent studies suggest a Vi-antigen translocation intermediate is accessible to glycanase digestion in the periplasm (53). This accessibility opens up the possibility of additional methods of diversification of EPS structure. For example, some O antigens exported by ABC transporters can be modified with side chain residues in the periplasm as they emerge from the exporter (60).

Capsular glycolipids emerging from the translocon may be retained in the outer membrane by the lipid anchor (54, 109), but ionic interactions with LPS molecules may also promote capsule organization and retention (37). Early microscopy studies of capsule growth following switch of *E. coli* K1 to permissive conditions revealed nascent CPSs emerging at a small number of randomly distributed sites on the cell surface (106). More sophisticated recent analyses show localized sites as nucleation points for capsule rafts, which may stay closely associated with OPX proteins (in synthesis/export complexes) (80). The rafts have limited diffusion mobility, and their expansion leads to a fully covered cell surface.

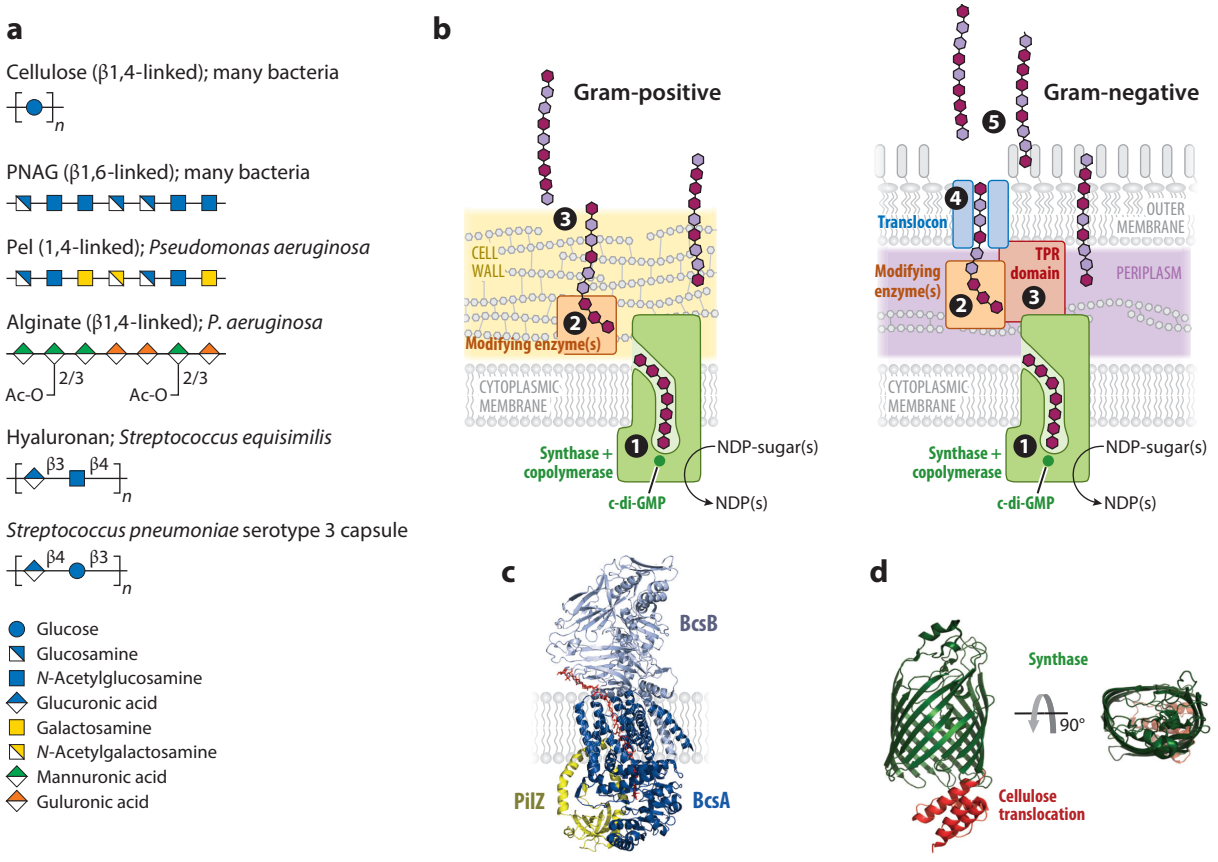


Figure 3

Synthase-dependent pathway. (*a*) Examples of repeat-unit structures of EPSs assembled by this strategy. (*b*) Hypothetical models for assembly and export in gram-positive and gram-negative bacteria. Steps ❶ and ❷ are conserved in both. (❶) Processive synthesis by a single glycosyltransferase catalytic site (in the absence of a lipid intermediate) drives translocation across the inner membrane. (❷) Processing of the nascent glycan by glycosidases and other modifying enzymes. In gram-negative bacteria, a TPR scaffold may direct the glycan to the translocon (❸), and it is translocated across the outer membrane (❹) by a β-barrel translocon. The TPR and translocon may be separate proteins or part of a single protein. In either system, the EPS can remain (noncovalently) associated with the cell surface or is released to the environment (❸ and ❺). (*c*) Solved structure of the BcsA (*dark blue*)-BscB (*light blue*) cellulose synthase complex from *Rhodobacter sphaeroides* (PDB 4HG6). The PilZ domain in BcsA is highlighted in yellow and the cellulose translocation intermediate in the channel is identified in red. (*d*) Solved structure of the *Escherichia coli* BcsC cellulose translocon with the first of its 19 TPR domains (PDB 6TZK). Abbreviations: Ac, acetyl; EPS, extracellular polysaccharide; PDB, Protein Data Bank; PNAG, poly-*N*-acetylglucosamine; TPR, tetratricopeptide repeat.

4. SYNTHASE-DEPENDENT SYSTEMS ASSEMBLE PROMINENT BIOFILM EXTRACELLULAR POLYSACCHARIDES

Synthases are processive GTs that produce EPSs frequently (but not exclusively) participating in biofilms and can protect pathogens against host defenses. The products are typically linear glycan backbones with one or two glycose components (**Figure 3*a***). Some bacteria have the genetic capacity to produce more than one synthase product (9). The cellulose pellicle formed at air-liquid interfaces in *Komagataeibacter* (formerly *Acetobacter*, *Gluconacetobacter*) *xylinus* cultures offers a classic biofilm example, recognized before the ubiquity of biofilms was appreciated. The high purity, nanofiber properties, and stability of bacterial cellulose (and cellulose composites) have led

to applications ranging from membranes and filters to drug delivery vehicles, wound dressings, and tissue scaffolds (reviewed in 85). Related EPSs from similar assembly strategies include β1,3-glucan (curdlan) from *Agrobacterium* spp., which has value in gelling applications (118), as well as mixed linkage β-glucans in rhizobia (85). Bacterial hyaluronan synthases generate a product that mimics mammalian glycosaminoglycans and offers opportunities in biomedical applications in drug delivery, tissue engineering, and device coating (18). Other synthase products with important roles in pathogenesis include alginates and hexosamine-rich polymers including poly-*N*-acetylglucosamine (PNAG) and Pel (59, 77). The latter provide rare examples where conserved biosynthesis genes are found in a range of gram-negative and -positive bacteria (9, 107).

Synthesis of these products typically occurs without a lipid acceptor and involves a transmembrane synthase-translocase complex (usually involving two proteins) coupling synthesis to export (**Figure 3*b***). In gram-negative bacteria, this complex is typically linked to a β-barrel outer membrane translocon via a periplasmic scaffold containing tetratricopeptide repeat (TPR) motifs (59). Well-characterized examples of these systems are regulated by the important signaling molecule cyclic-3′,5′-GMP (c-di-GMP) (102), but activation mechanisms vary between representatives.

4.1. Synthase-Translocase Catalytic Cores

Cellulose synthase is an influential prototype and offers the only crystal structure for the functional core enzyme (BcsAB from *Rhodobacter sphaeroides*; reviewed in 63) (**Figure 3*c***). In the presence of the c-di-GMP activator, these proteins are sufficient for cellulose synthesis in vitro. BcsA contains a UDP-Glc-dependent GT2 enzyme that retains the nascent glycan in a translocation channel that accommodates ∼10 residues during processive chain extension. BcsB is predominantly periplasmic and contains two carbohydrate-binding modules that may assist in export. c-di-GMP binding to a PilZ domain at the C terminus of BcsA drives conformational changes that retract a gating loop from the active site to modulate GT activity. Structures in inactive and c-di-GMP-activated states, as well as elegant studies of a reaction cycle performed in crystallo, provided unprecedented insight into the coordination of synthesis and export (68, 69). UDP-Glc binding and polymer extension are accompanied by coordinated changes in positioning of the gating loop and the finger helix, which contacts the nonreducing terminal Glc in the product and is correlated with stepwise translocation. The core synthase-translocase is accompanied by additional accessory proteins that can vary in different species (9, 85). In *E. coli*, they contribute to a larger inner membrane macrocomplex (42), but insight into the exact roles of each accessory protein is limited; they are implicated in assembly and stability of the complex.

The extent of current variations in synthase structure and activation has been reviewed recently (59). While enzymes containing GT modules are evident in each case, the systems differ in the number of proteins, their organization, and their mode of regulation by c-di-GMP. For example, in *P. aeruginosa* alginate and Pel biosynthesis, c-di-GMP binds to the noncatalytic partner by different mechanisms. As might be expected, the different modes of regulation have been proposed to impact different steps in the synthetic process.

4.2. Translocation Across the Periplasm and Outer Membrane

In gram-negative bacteria, translocation of synthase products across the periplasm and outer membrane is typically dependent on two structural elements: a TPR-rich polypeptide and a 16–18-stranded β-barrel channel (reviewed in 59). These can exist as separate polypeptides (e.g., alginate biosynthesis) or be part of a single protein (e.g., cellulose, Pel, PNAG biosynthesis). The overall objective is the same as that of Wzy- and ABC transporter–dependent systems, but the translocation machinery is clearly distinct.

In the cellulose translocon (BcsC) (**Figure 3**), only the extracellular side of the large (15 Å) channel is occluded, while the example from the alginate system (AlgE) is gated at the extracellular and periplasmic faces (96). Gating provides a mechanism to retain the integrity of the outer membrane size-exclusion barrier in the absence of active export. A short helical linker leads from the last TPR of BcsC into the first β strand of the barrel, and an electropositive pocket that is conserved (and functionally important) in comparable systems (1). Aromatic residues in the lumen may participate in translocation by ring stacking and H bonding. The 19 TPR motifs of BcsC create a flexible superhelix (74) sufficient to reach the substantial periplasmic domain of BcsB. Similarly, the alginate TPR protein (AlgK) structure shows a compact superhelical structure that is known to promote the proper localization of the translocon (AlgE) (41), and its interaction with the synthase (Alg8/Alg44) is required for polymerization (84). Transit through the translocon may be driven by the polymerization reaction, with the TPR domain orienting the nascent polymer emerging from the synthase complex and opening the periplasmic gate in the translocon (96). The physical properties of the glycan products make them particularly prone to aggregation or interaction with other cellular components, so a coordinated exit route physically coupling synthesis to translocation seems logical. However, it is unknown how the TPR domains interact with the glycan in transit, or how the final step of polymer secretion is accommodated if it requires release from the synthase catalytic site and loss of the potential driving force.

While *R. sphaeroides* produces amorphous cellulose, other bacteria produce a crystalline product with commercial value, and the arrangement of synthetic complexes has been implicated in forming the higher-order structure (23, 63). In *K. xylinus*, nascent cellulose fibrils emerge from multiple sites along the lateral cell surface and assemble into higher-order microfibrils extending from the cell poles (8), while secreted cellulose localizes to the poles (48). The observed multimerization of synthase complexes may facilitate the formation of cellulose microfibrils from individual glycans assembled by adjacent complexes. Working models invoke a surface bundling of emerging glycan strands or bundling within the periplasm prior to passage through the same translocon.

4.3. Periplasmic Modifications

A frequent feature in synthase-dependent pathways is the introduction of structural modifications following polymerization (108). Unlike classical cellulose producers such as *K. xylinus*, *Pseudomonas fluorescens* acetylates its cellulose (93), while *E. coli* and other *Enterobacteriaceae* modify ∼50% of Glc residues with phosphoethanolamine residues derived from phosphatidylethanolamine (94, 98). Other processes include conversion of ManA residues to GulA and O-acetylation in alginate by the 5′-epimerase (AlgG) (111) and the *O*-acetyltransferases (AlgJ, AlgX), respectively (4). Pel and PNAG are partially de-N-acetylated by PelA and IcaB/PgaB, respectively, to generate a heterogeneous backbone that, depending on the organism, can influence the synthesis, translocation, localization, and adhesiveness of the product (reviewed in 77). Another common feature in gram-negative examples is a periplasmic glycanase. Pel and PNAG incorporate glycosyl hydrolase and de-*N*-acetylase domains into the same (PelA and PgaB) polypeptides, while the cellulose and alginate machineries include a stand-alone glycosyl hydrolase (BcsZ), or lyase (AlgL) (59). Some of these glycanases have potential applications in strategies to eradicate biofilms where the corresponding EPS plays a prominent role (77). In this context, addition of exogenous enzyme degrades the organizing EPS to compromise the integrity of the biofilm and diminish its ability to protect the bacteria within. Overarching concepts in the structural arrangement of the periplasmic components are not yet established, but some interesting insights have been gained. PelA activity is enhanced by binding the TPR domain of PelB (62). PgaB is an outer membrane lipoprotein, and molecular dynamics simulations suggest the nascent glycan remains associated

with the enzyme as it moves between the two catalytic domains (57), facilitating a sequential modification process (58). The absence of a contained periplasm in gram-positive bacteria presents a challenge in retaining modifying enzymes; as an example the PNAG-modifying IcaB enzyme appears to associate with the membrane via a hydrophobic loop (56). The glycanases have been implicated in the formation of cellulose microfibrils, generation of products with particular chain length distributions, degradation of off-pathway glycans, and export (reviewed in 59). The conservation of modification systems in gram-positive and -negative bacteria seems to argue against a role confined to export.

4.4. An Atypical Synthase in *S. pneumoniae* Serotype 3

While the most synthase products are synthesized without a lipid carrier, the type 3 pneumococcal synthase provides one exception (reviewed in 116). This glycan is assembled on a phosphatidylglycerol acceptor. While the polymerization reaction has been investigated at a biochemical level, no structural information is available to offer insight into how this enzyme accommodates the lipid during translocation.

5. EXTRACELLULAR TRANSGLYCOSYLASES PRODUCING BACTERIAL DEXTRANS

The final EPS assembly strategy is conceptually the simplest because it relies on a secreted enzyme, dispensing with the need for donor synthesis and export processes. Glucansucrase enzymes utilize sucrose or starch compounds to generate a range of α-linked glucans, while fructansucrases assemble β-linked fructans (28). The EPS products are prevalent in lactic acid bacteria and often function in adhesion and colonization of surfaces, highlighted by *Streptococcus mutans* sucrose-dependent dental plaque biofilms (7). The glycans can possess linear backbones but may also be interconnected by nonstoichiometric interchain linkages to generate large, nonuniform structures of varying sizes. Variations in size (from short oligosaccharides to $>10^6$ Da) and degrees of branching lead to properties important in commercial applications, ranging from food thickeners to anticoagulants (121).

Glucansucrases are particularly well characterized (reviewed in 28, 115). Some bacteria are able to express multiple enzymes generating EPSs with varying properties, which contribute in different ways to the biology of the organism (e.g., plaque formation). Other enzymes are individually capable of generating branched polymers with different linkage types. These enzymes resemble glycosyl hydrolases from the GH13 and GH70 families and catalyze transglycosylation reactions using a retaining mechanism. Glucansucrases are large enzymes (120–220 kDa) possessing a variable N-terminal domain that may be involved in association with the cell wall but is not required for activity. The catalytic domains contain three structural elements (A–C), where the A domain is a circularly permutated form of a catalytic $(\beta/\alpha)_8$ barrel typical of glycosyl hydrolases, accompanied by two additional motifs (IV and V) unique to the glucansucrases. The latter are proposed to move bound glucans toward, or away from, the catalytic site and influence product size. Mutations in acceptor binding sites influence the spectrum of linkages in the EPS, as well as the size distribution of the polymer.

6. FUTURE PROSPECTS

The last decade has seen impressive advances in understanding EPS biosynthesis, due to improved analytical methods for determining the structures of intermediates and products, as well as the combined application of new approaches to investigate protein structure and function. Critical protein classes have been investigated to various extents in key prototypes, but the preceding text

highlights important gaps. The extent of conservation in these proteins will support a functional understanding of new systems and reveal new variations on the existing pathways, as they arise from genomics data. In the authors' opinion, there are two immediate challenges for the field. One is to understand the structure and organization of functional assembly/export heterocomplexes, and the other is to identify factors that underpin the cellular distribution, localization, and activities of those complexes during the cell cycle.

Advances in understanding the principles in EPS assembly and export offer new possibilities for biopolymer applications by versatile and powerful glycoengineering (see 89). Whereas improvements in commercial production once depended on varying culture conditions and selecting mutants with desirable properties, current knowledge and tools allow precisely targeted approaches. Metabolomics/systems biology insight can be exploited to strategically alter pools of donor substrates to improve yields, while precise mutations can change residues that influence enzyme specificity, affect polymer chain length, or add expendable glycan substructures. Novel structures can be generated by introducing genes encoding new functionalities, and chemi-enzymatic synthesis can generate controlled homogeneous products. Glycoprotein engineering has already successfully exploited the shared involvement of Und-PP-intermediates in different glycoconjugate assembly systems. In the current context, in vivo protein glycosylation to create EPS-targeted neoglycoprotein vaccines for *S. pneumoniae* (31) and *K. pneumoniae* (25) provide excellent examples. However, broad opportunities in diagnostics and other biomedical applications are now enabled.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by funding awarded to C.W. by the Canadian Institutes of Health Research (Foundation Grants Program) and the Canadian Glycomics Network. C.W. is a recipient of a Canada Research Chair, and C.S. holds a CIHR Canada Graduate Scholarship. The authors gratefully acknowledge lab members past and present for help with figures and comments on the draft. We acknowledge the many scientists whose contributions in this field could not be cited due to limited space.

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Annual Review of Microbiology
Volume 74, 2020

Contents