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Synthase-dependent exopolysaccharide secretion in Gram-negative bacteria

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Abstract

The biosynthesis and export of bacterial cell-surface polysaccharides is known to occur through several distinct mechanisms. Recent advances in the biochemistry and structural biology of several proteins in synthase-dependent polysaccharide secretion systems have identified key conserved components of this pathway in Gram-negative bacteria. These components include an inner-membrane-embedded polysaccharide synthase, a periplasmic tetratricopeptide repeat (TPR)-containing scaffold protein, and an outer-membrane β-barrel porin. There is also increasing evidence that many synthase-dependent systems are post-translationally regulated by the bacterial second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP). Here, we compare these core proteins in the context of the alginate, cellulose, and poly-β-D-*N*-acetylglucosamine (PNAG) secretion systems.

Keywords

synthase; exopolysaccharide; alginate; cellulose; poly-β-D-*N*-acetylglucosamine

Role of exopolysaccharide production

The secretion of polysaccharides by bacteria is a physiological process that occurs under a multitude of different environmental circumstances. The role of these polysaccharides includes basic functions such as maintaining the structural integrity of the cell envelope and preventing cellular desiccation, as well as more complex functions such as facilitating interactions within bacterial communities, and between bacteria and eukaryotes. Bacterial polysaccharide production plays a direct role in human health, in part because of the ability of many pathogenic bacteria to form multicellular conglomerates called bio-films. The structural integrity of bacterial biofilms is highly dependent on a self-produced extracellular

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Note added in proof

Since this paper first appeared online (30 October 2012), the structure of the BcsA/BcsB inner membrane cellulose synthase complex has been determined (PDB 4HG6 [97]), and evidence that PgaC and PgaD interact with each other, and c-di-GMP to form a functional PNAG synthase complex reported [98]. The conclusions of these two studies provide strong support for the mechanisms of cellulose and PNAG secretion proposed herein.

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matrix that may comprise exopolysaccharides, nucleic acids, and proteins [1–3]. The major components of the biofilm matrix differ, depending on the bacterial species and strain, the stage of biofilm development, and the environmental conditions. In *Pseudomonas aeruginosa*, a model organism that has been extensively used to study biofilm development, the exopolysaccharide component of the biofilm matrix predominates and protects the bacteria from host defense mechanisms and administered antibiotics [4–7]. Exopolysaccharides produced by bacteria also have industrial applications in the paper, food, and health industries, and thus an understanding of how these long polymers are secreted may lead to further efficiencies in commercial polysaccharide production.

Secretion of cell-surface polysaccharides in Gram-negative bacteria

Despite the enormous chemical diversity among the carbohydrate building blocks of bacterial exopolysaccharides, the molecular mechanism by which these biopolymers are assembled and exported from the cell can currently be categorized into three distinct mechanisms (Figure 1). These include the Wzx/Wzy- and ATP-binding cassette (ABC) transporter-dependent pathways, both of which use a lipid acceptor to initiate polysaccharide synthesis, and a synthase-dependent pathway, for which the requirement for a lipid acceptor molecule depends on the polysaccharide in question. In Wzx/Wzy-dependent secretion systems, such as *Escherichia coli* group 1 capsular polysaccharides (CPS) and lipopolysaccharide (LPS) O-antigen, the polysaccharide repeat unit is assembled on an undecaprenyl phosphate acceptor moiety by various inner-membrane-embedded or -associated glycosyl transferases [8,9]. This synthesized precursor is then transported across the inner membrane by a flippase, Wzx [10–12], before being polymerized into a high-molecular-weight polysaccharide by the periplasmic polymerase Wzy [13]. In comparison, the ABC transporter-dependent systems, such as *E. coli* group 2 CPS and LPS common antigen, assemble the entire polysaccharide chain on a lipid acceptor, the identity of which varies depending on the polysaccharide being synthesized, before transporting the polymer across the inner membrane via an ABC transporter [14–16]. Despite these mechanistic differences in their modes of polysaccharide assembly, both the Wzx/ Wzy- and ABC transporter-dependent secretion systems use similar protein families to facilitate exopolysaccharide export across the periplasm and through the outer membrane. This process involves proteins from the outer-membrane polysaccharide export (OPX) and polysaccharide copolymerase (PCP) protein families [17,18]. The X-ray crystal structures of *E. coli* Wza [19], an OPX protein, and several PCPs [20,21] have demonstrated that both of these proteins arrange into homo-oligomeric assemblies that interact with one another to form a protein channel through which the nascent polysaccharide chain is exported from the cell [22].

Synthase-dependent exopolysaccharide secretion can occur in the presence or absence of a lipid acceptor molecule, depending on the polysaccharide [23–25]. In these systems, it appears that a membrane-embedded glycosyl transferase is able to facilitate simultaneous polymer formation and translocation across the inner membrane [25]. In some of the better-characterized Gram-negative synthase-dependent secretion systems such as *P. aeruginosa* alginate and *Gluconacetobacter xylinus* cellulose, polymerization is also regulated by an inner-membrane receptor, sometimes referred to as a co-polymerase, that binds the bacterial

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second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) to activate polysaccharide production [26,27]. Once the polymer reaches the periplasm, a tetratricopeptide repeat (TPR)-containing scaffold protein is thought to protect it from degradation before it is exported across the outer membrane through a β-barrel porin [28–30]. This export apparatus requires protein families that are clearly distinct from the OPX and PCP proteins used by the Wzx/Wzy- and ABC transporter-dependent pathways, and given the predictability of these protein domain families by sequence analysis programs such as SMART [31,32] and Phyre^2 [33], they serve as identifiable hallmarks of synthase-dependent exopolysaccharide secretion systems in Gram-negative bacteria. Here, we summarize the current understanding of synthase-dependent polysaccharide production in the context of the secreted exopolysaccharides alginate, cellulose, and poly-β-D-*N*-acetylglucosamine (PNAG).

Synthase-dependent production of alginate

Alginate is a random linear polymer of 1,4-linked β-D-mannuronic acid and its C5 epimer α-L-guluronic acid [(β-D-ManUA-(1→4)-β-L-GulUA)*n*], which was originally identified in brown seaweeds and subsequently in several species of Gram-negative bacteria [34–36]. Interest in alginate biosynthesis has been driven by the observation that mucoid isolates of the opportunistic pathogen *P. aeruginosa* found in the lungs of cystic fibrosis (CF) patients secrete copious amounts of the polysaccharide [37]. Moreover, conversion of *P. aeruginosa* from a non-mucoid to a mucoid phenotype correlates with poor prognosis among these patients [38,39]. Current understanding of the mechanism of alginate biosynthesis and secretion has been gained from studies of the alginate-producing bacteria *P. aeruginosa* and *Azotobacter vinelandii*. In these organisms, alginate is first synthesized as a homopolymer of 1,4-linked β-D-mannuronic acid before being epimerized at the polymer level to form the mature polysaccharide [40,41]. In addition, it has been found that the O2 and/or O3 positions of β-D-mannuronic acid residues in bacterial alginates can be acetylated [42,43], which increases the water-binding capacity of the polymer and, during infection, protects *P. aeruginosa* from opsonic phagocytosis by the immune system of the host [44,45]. Moreover, *O*-acetylated alginate has been identified as an important structural component of biofilms produced by mucoid strains of *P. aeruginosa* [46].

The proteins responsible for the polymerization and export of alginate are encoded on the *algD* operon (Figure 2). Synthesis of poly-β-D-mannuronic acid from GDP-mannuronic acid occurs at the inner membrane and requires the integral inner membrane proteins Alg8 and Alg44 (Figures 3 and 4; Table 1). Alg8 is a putative alginate synthase because it contains five predicted trans-membrane domains and a large cytoplasmic synthase domain that shares homology with family 2 glycosyl transferases [47,48]. The polymerization reaction also requires the c-di-GMP-binding activity of the inner-membrane protein Alg44 [26]. Alg44 is a single-pass transmembrane protein that is predicted to have an N-terminal cytoplasmic c-di-GMP-binding PilZ domain and C-terminal periplasmic region that may resemble a membrane fusion protein (MFP) domain [47]. It is thought that these two proteins act in concert to facilitate the polymerization and export of poly-β-D-mannuronic acid across the inner membrane by a mechanism that is regulated by c-di-GMP binding to Alg44. This hypothesis was derived, in part, from the observation that site-specific mutations that

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compromise the c-di-GMP-binding ability of Alg44 abrogate alginate production *in vivo* [26]. The role of MFP domains as periplasmic adaptors that join inner-membrane transport proteins to outer-membrane export proteins in bacterial tripartite drug-efflux pumps suggests that the MFP domain of Alg44 may take part in periplasmic protein–protein interactions (Figure 3) [49]. At present, there are no experimental data confirming that Alg8 and Alg44 are directly responsible for export of the newly synthesized polymer across the inner membrane or if the MFP domain of Alg44 couples alginate polymerization to its export.

Once in the periplasm, poly-β-D-mannuronic acid is epimerized by the polymer-level mannuronan C5 epimerase AlgG [40]. AlgG is thought to play dual roles in both C5 epimerization and secretion of the nascent alginate polymer across the periplasm [50]. An *algG* deletion mutant produces degraded alginate fragments that are characteristic of digestion products of the periplasmic alginate lyase AlgL, suggesting that AlgG also plays a role in protecting the alginate polymer as it traverses the periplasm [50].

The *algI*, *algJ*, and *algF* gene products have all been implicated in the *O*-acetylation of mannuronic acid residues [51,52]. AlgI and AlgJ are integral inner-membrane proteins, whereas AlgF localizes to the periplasm. Topology modeling of AlgI suggests that it contains at least seven transmembrane helices, whereas except for a single trans-membrane helix at its N-terminus, the majority of AlgJ resides in the periplasm [53]. The current model of alginate *O*-acetylation involves export of an unknown cytoplasmic acetyl donor by AlgI across the inner membrane to the periplasm, where the acetate group is then transferred to alginate by the activities of AlgJ and AlgF [54]. AlgX is a periplasmic protein of unknown function; however, its high sequence similarity to AlgJ suggests that it may also play a role in alginate *O*-acetylation [55,56]. As with the *algG* deletion mutant, an *algX* deletion mutant produces AlgL-degraded alginate, suggesting that AlgX may also play a role in guiding the mature alginate polymer across the periplasm [55].

The TPR-containing outer-membrane lipoprotein AlgK is believed to guide the mature polymer towards the integral outer-membrane protein AlgE, which facilitates translocation of alginate across the outer membrane [29,30]. The proposed function of AlgK was derived, in part, from phenotypic studies demonstrating that in the absence of AlgK, alginate is degraded by AlgL [28,57,58]. The presence of TPRs in the structure of AlgK suggests that it may serve as a scaffold to which the other periplasmic Alg proteins interact to form a multiprotein complex (Figure 3) [29]. TPR-containing proteins often function as protein–protein interaction modules and are involved in a variety of different cellular processes in all domains of life [59]. In Gram-negative bacteria, TPR domains facilitate protein–protein interactions within the outer-membrane β-barrel assembly machinery (BAM) complex, and between the chaperone and substrate for several translocator proteins exported by the type III secretion system (T3SS) apparatus [60,61].

The recent X-ray crystal structure of AlgE shows that it adopts an 18-stranded β-barrel with a highly electropositive interior (Figure 3) [30]. Given the strict conservation of many of the pore-forming residues, it has been suggested that AlgE forms a highly specific translocation pathway for the negatively charged alginate polymer. On the basis of subcellular fractionation experiments using an *algK* deletion mutant, it has also been proposed that

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AlgE interacts directly with AlgK because a significant proportion of AlgE mislocalizes to the inner membrane in this mutant [29]. Lending further support to this hypothesis is the prediction that the outer-membrane proteins involved in cellulose and PNAG secretion (discussed in the following sections) each form large two-domain proteins with a periplasmic TPR-containing domain and a transmembrane β-barrel (Figure 4) [30].

At present, there is a lack of definitive evidence that the alginate biosynthesis and export proteins interact to form a *trans*-envelope complex, although initial studies showing that *in vitro* alginate polymerization requires both inner-and outer-membrane fractions suggest that this may be the case [48]. Preliminary evidence demonstrating direct interactions between AlgX, AlgK, and the regulatory protein MucD has also been reported recently [62]. Although an AlgK–AlgX interaction is logical given the involvement of the two proteins in the alginate secretion process, MucD is a homolog of *E. coli* HtrA/DegP and a negative regulator of alginate biosynthesis [63]. The proposed function of MucD is to degrade cell-wall stress signals that activate transcription of the *algD* operon [64]. The proposed role of MucD is to function upstream of *algX* and *algK* translation, so the biological significance of the MucD–AlgX–AlgK complex observed *in vitro* is unclear at this point. Although the individual functions of the proteins involved in the polymerization, modification, and export of alginate have been elucidated, future research needs to address which of these proteins interact with one another to facilitate the overall synthesis and secretion process.

Synthase-dependent production of cellulose

Produced by higher plants, fungi, algae, and bacteria, the β-1,4-linked D-glucose homopolymer cellulose [(β-D-Glc-(1→4)-β-D-Glc)*n*] is one of the most abundant polysaccharides found in nature. Despite this, little is known about the molecular mechanism of its biosynthesis and export. In bacteria, cellulose biosynthesis was first described in *G. xylinus* (formerly *Acetobacter xylinum*) and has since been described in a variety of Gram-negative bacteria including *E. coli*, *Salmonella enterica*, and *Vibrio fischeri* among others (Figure 2) [65–68].

Although the order of genes required for cellulose production exhibits more variability among bacteria compared to those involved in alginate production, the same core protein components involved in its biosynthesis and export appear to be conserved between the two polysaccharide secretion systems (Figures 2 and 4; Table 1). For example, the *E. coli bcsA* gene (*acsA* in *G. xylinus*) encodes the cellulose synthase protein. Similarly to the alginate synthase Alg8, BcsA is an inner-membrane protein with multiple transmembrane domains and a cytoplasmic family 2 glycosyl transferase domain [67,69]. BcsA is thought to both catalyze cellulose polymerization from UDP-glucose and facilitate translocation of the newly formed polymer across the inner membrane. In addition, BcsA contains a PilZ domain at its C terminus, whose c-di-GMP binding activity activates cellulose production [27]. Thus, unlike alginate secretion, in which Alg8 and Alg44 are responsible for the polymerization and c-di-GMP binding activities, respectively, in cellulose biosynthesis both of these functions are carried out by a single protein (Figures 2 and 4). The *bcsB* gene product localizes to the inner membrane and is required for both *in vitro* and *in vivo* cellulose production; however, its specific role in cellulose production is unclear at this point

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[70]. It has been proposed that the *bcsC* gene encodes a large outer-membrane protein that contains an N-terminal TPR-containing domain that resides in the periplasm and a C-terminal porin domain that facilitates cellulose export across the outer membrane [29]. Thus, it is thought that BcsC contains a domain architecture that resembles AlgK and AlgE from the alginate secretion system [30]. This putative function would also explain why BcsC is necessary for cellulose production *in vivo* but is dispensable for its production *in vitro* [70]. The *bcsZ* gene is located within the cellulose biosynthetic operon in some bacteria or elsewhere in the genome in others. *bcsZ* encodes a periplasmic enzyme with endo-β-1,4-glucanase activity that may be required for degradation of accumulated cellulose in the periplasm and/or cleavage of nascent cellulose chains to allow micro-fibril formation to occur outside the cell [71]. BscQ, which is homologous to the *E. coli* cell division protein MinD, localizes to the cell pole and thus may be required for polar localization of the cellulose biosynthesis apparatus in *E. coli*, *S. enterica*, and *Burkholderia cenocepacia* [72]. However, it should be noted that the authors of this study were not able to detect polar localization of the other Bcs proteins even though cellulose itself was produced at the cell pole. The AcsD protein, which is unique to *G. xylinus*, arranges into a homo-octomeric assembly that is capable of binding cellulose [73]. It is thought that the AcsD multimer exists extracellularly and that its function is to twist the newly synthesized glucan polymers into higher-order cellulose fibrils [73,74]. This hypothesis would also help to explain why *G. xylinus* produces fibrillar cellulose whereas other bacteria that lack an AcsD homolog, such as *E. coli*, produce amorphous cellulose.

P. fluorescens SBW25 and *Pseudomonas syringae* patho-var *tomato* DC3000 produce an acetylated form of cellulose whose production requires the *wss* operon (Figure 2) [75,76]. This operon is predicted to encode not only proteins involved in the secretion of non-acetylated cellulose (WssA/ BcsQ, WssB/BcsA, WssC/BcsB, WssD/BcsZ, and WssE/ BcsC) but also proteins that resemble those involved in the *O*-acetylation of alginate (WssG/ AlgF, WssH/AlgI, and WssI/AlgJ) (Figure 4 and Table 1). WssF is also involved in the *O*-acetylation of cellulose; however, there does not appear to be a functionally similar protein involved in alginate *O*-acetylation [75]. In addition, the *wss* operon of *P. fluorescens* SBW25 contains a second MinD homolog, WssJ; it is speculated that WssJ plays a role in cellular localization of the cellulose *O*-acetylation proteins, as opposed to the cellulose synthase proteins, whose localization is more likely to be regulated by WssA given its similarity to BcsQ [75]. However, characterization of the acetylated cellulose produced by these bacteria is still in its infancy and requires experimental confirmation of the proposed functions for each of the proteins.

Synthase-dependent production of PNAG

The genes responsible for synthesis of PNAG have recently been identified in a number of Gram-negative bacteria including *E. coli*, *Yersinia pestis, Actinobacillus pleuropneumoniae*, and *Bordetella bronchiseptica*, among others (Figure 2) [77–80]. PNAG is a β-1,6-linked *N*-acetyl-D-glucosamine homopolymer [(β-D-GlcNAc-(1→6)-β-D-GlcNAc)n] that functions as an important component of the biofilm matrix produced by these organisms and thus contributes to their overall persistence during infection. Unlike alginate and cellulose, which are synthesized as non-acetylated polymers by their respective synthases and subsequently

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O-acetylated in the periplasm, PNAG is assembled as a fully *N*-acetylated precursor starting from a UDP-*N*-acetylglucosamine precursor. The PgaC protein (*Y. pestis* HmsR and *B. bronchiseptica* BpsC), similar to Alg8, BcsA, and WssB, is predicted to contain multiple transmembrane domains and a large cytoplasmic domain that shares homology with family 2 glycosyl transferases (Figure 4). Therefore, PgaC is a putative PNAG synthase that is thought to catalyze the polymerization of β-1,6-linked *N*-acetyl-D-glucosamine and facilitate its export across the inner membrane. The precise function of the predicted inner-membrane protein PgaD/ HmsS/BpsD is unknown; however, it is thought to play a role in the polymerization process because, similar to PgaC, its deletion abrogates the production of PNAG [81]. Although it is not predicted to contain a PilZ domain, the possibility that PgaD binds c-di-GMP to post-translationally regulate PNAG production cannot be ruled out given the diversity of protein folds that are capable of binding to the dinucleotide [82]. Once in the periplasm, the PNAG polymer is partially de-*N*-acetylated by the carbohydrate esterase PgaB/HmsF/BpsB. A recent structure–function characterization of *E. coli* PgaB has shown that it harbors a de-*N*-acetylase domain with low catalytic efficiency [83]. These observations corroborate the relatively low amount (~22%) of de-*N*-acetylation observed *in vivo* and has led to speculation that the differing amounts of de-*N*-acetylation observed among PNAG-producing bacteria are probably related to the enzyme activity of their PgaB homolog. PgaA is a predicted outer-membrane protein that is proposed to have a similar domain arrangement as BcsC/AlgK and AlgE. In the absence of PgaB, PNAG accumulates in the periplasm of *E. coli*, leading to speculation that the putative export function of PgaA is specific for partially de-*N*-acetylated (mature) PNAG [81]. Aside from PgaB, the proteins involved inPNAG polymerization and export have not been functionally characterizedtodate. Structural and functional characterization of the PgaA, PgaC, and PgaD proteins will be required to fully understand the mechanism of PNAG secretion across the cell envelope.

Additional synthase-dependent polysaccharide secretion systems

Alginate, cellulose, and PNAG are the most studied synthase-dependent secretion systems in Gram-negative bacteria, but additional polysaccharides appear to use a similar mechanism of biosynthesis and export. *Pasteurella multocida* produces hyaluronan (HA), which comprises repeating units of D-glucuronic acid and *N*-acetyl-D-glucosamine [(1→4)-β-D-GlcUA-(1→3)-β-D-GlcNAc-(1→4)] and requires hyaluronan synthase (HAS) for its production [84,85]. This synthase is the fusion of two glycosyl transferases, one with specificity towards β-D-*N*-acetylglucosamine and the other towards β-D-glucuronic acid. The details of HA export through the periplasm and across the outer membrane are unclear at this point so it is difficult to ascertain if the same protein components as for alginate, cellulose, and PNAG are utilized. There is also controversy as to whether HA assembly requires a lipid precursor. However, a recent study on a HAS from the Gram-positive bacterium *Streptococcus equisimilis* indicates that the polymerization and membrane translocation processes do not require a lipid-linked precursor in this bacterium [25].

The PEL polysaccharide, whose chemical structure has yet to be identified, is produced by some strains of *P. aeruginosa* and *Ralstonia solanacearum* [86]. Similar to alginate and cellulose, PEL is regulated post-translationally by c-di-GMP [87]. However, this regulation occurs via the degenerate GGDEF domain of PelD and not a PilZ domain as in alginate and

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cellulose production [88]. Therefore, the mechanism by which PelD exerts its regulation may differ from the PilZ domains of Alg44 and BcsA. In addition, the putative PEL polymerase PelF is predicted to localize to the cytoplasm and is thus unlikely to facilitate polysaccharide translocation across the inner membrane as has been proposed for Alg8 and BcsA [89]. At present, characterization of the Pel proteins is still in its early stages and it remains to be determined if PEL polysaccharide assembly occurs on a lipid carrier.

Curdlan [β-D-Glc-(1→3)-β-D-Glc)*n*] production by *Agro-bacterium tumefaciens* requires the curdlan synthase protein CrdS, which probably uses a synthase-dependent pathway given its sequence similarity to cellulose synthases [90]. However, lack of experimental characterization of this system makes it difficult to determine its mechanism of biosynthesis and secretion.

Concluding remarks

Although this review highlights some interesting commonalities between the better-studied synthase-dependent exopolysaccharide secretion systems, it is clear that more research is required to tease out the molecular details of this process. One central question that has yet to be addressed is whether or not the synthase-dependent secretion systems highlighted here form a *trans*-envelope multiprotein complex as observed in the Wzx/Wzy- and ABC-transporter-dependent systems [22]. Although this hypothesis seems feasible for alginate and cellulose secretion, for which there are a significant number of proteins in the periplasm that could facilitate the formation of such a complex, the apparent simplicity of the PNAG secretion system suggests that it may not have enough protein components to span the ~200-Å periplasmic space unless a constriction occurs [91].

Post-translational regulation by c-di-GMP is another process that appears widespread among synthase-dependent polysaccharide systems. However, the mechanism by which c-di-GMP exerts its effect has not been addressed for any of these systems. Is binding of c-di-GMP to its receptor a switch that stimulates the catalytic activity of its associated synthase or does it also serve to couple exo-polysaccharide polymerization to export through alteration of protein–protein interactions by an as yet unidentified allosteric mechanism? Moreover, how does the synthase itself facilitate extrusion of the nascent polymer across the inner membrane? To date, the only synthase whose polysaccharide export activity has been definitively shown is the aforementioned bacterial HAS from *S. equisimilis.* To address these questions and many others (Box 1), *in vitro* reconstitution of these systems using purified components will be required. High-resolution crystal structures of these components will also be critical, because they will help in generating mechanistic hypotheses that can then be tested experimentally.

Box 1

Outstanding questions

- How is synthase-dependent exopolysaccharide polymerization initiated? If, as proposed, some synthase-dependent secretion systems do not require a lipid acceptor molecule, then what molecular entity serves as the nucleophile for

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the first sugar–nucleotide precursor molecule? As has been shown for some UDP-glucosyl transferases [94], is water capable of functioning as a sugar–nucleotide acceptor?

- By what mechanism does c-di-GMP post-translationally regulate exopolysaccharide polymerization? Does c-di-GMP binding to its target receptor alleviate inhibition of its associated synthase or does it allosterically activate synthase activity through conformational changes? Does c-di-GMP binding also serve to couple exopolysaccharide polymerization to export through protein–protein interactions?
- How does the exopolysaccharide cross the cytoplasmic inner membrane? Are the transmembrane domains of the synthase sufficient for inner membrane transport of the polymer [25] or are additional protein components required? As described above, what is the role of c-di-GMP binding in this process?
- In the alginate and acetylated cellulose systems, what is the chemical identity of the acetyl donor? Mechanistically, how is the acetate group transported across the inner membrane and transferred to the nascent polymers? Identification of this acetyl donor may also shed light on the mechanism of *O*-acetylation in other polysaccharides such as bacterial (peptidoglycan) and plant (xyloglucan) cell-wall polymers [95,96].
- Do the inner and outer membrane components of synthase-dependent polysaccharide secretion systems interact with one another to form a *trans*-envelope complex as observed for OPX and PCP proteins of the Wzx/Wzy-dependent polysaccharide secretion systems?

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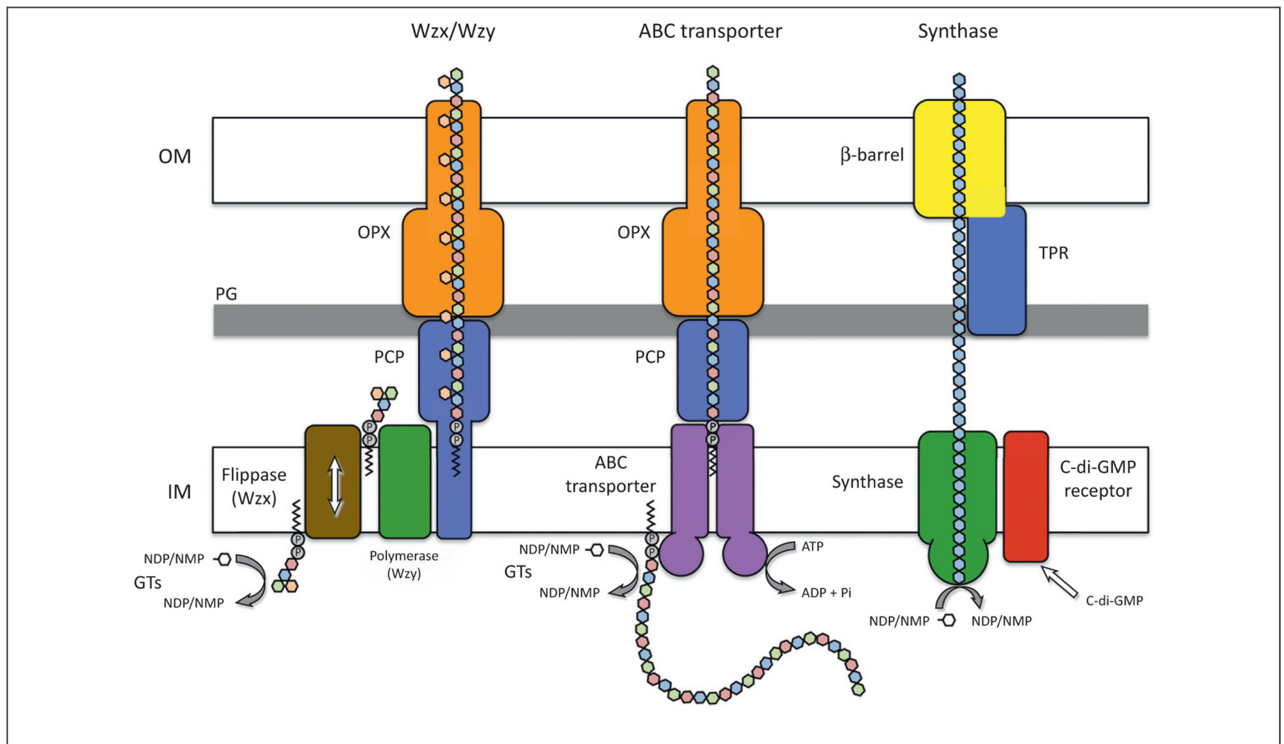

Figure 1.

Mechanisms of polysaccharide secretion. Cartoon schematic of the Wzx/Wzy-, ABC-transporter- and synthase-dependent pathways for exopolysaccharide biosynthesis and export. The key protein components for each pathway are indicated on the diagram. Glycosyl transferases (GTs) synthesize the lipid-linked polysaccharide repeat units from nucleotide diphosphates (NDPs) or nucleotide monophosphates (NMPs) in the Wzx/Wzy- and ABC-transporter-dependent systems. For Wzx/Wzy-dependent secretion, the polysaccharide repeat unit is assembled on an undecaprenyl phosphate carrier located in the inner leaflet of the inner membrane before being transported across the inner membrane by the flippase Wzx. In the periplasm, the repeat units are assembled into the mature polysaccharide by the polymerase Wzy before being exported through the periplasm and across the outer membrane by a translocation pathway formed by members of the PCP and OPX families of proteins. For ABC transporter-dependent secretion, the entire polysaccharide is assembled on a lipid carrier located in the inner leaflet of the inner membrane before being transported across the inner membrane by an ABC transporter. The polysaccharide is then exported through the periplasm and across the outer membrane by a translocation pathway formed by members of the PCP and OPX families of proteins. For synthase-dependent secretion, the polysaccharide is polymerized and exported across the inner membrane by an inner-membrane synthase protein. In some instances, the activity of the polysaccharide synthase is post-translationally regulated by an inner-membrane c-di-GMP receptor. The polysaccharide is then exported across the outer membrane by a periplasmic TPR-containing protein and an integral outer-membrane β-barrel. Abbreviations: OPX, outer membrane polysaccharide export; PCP, polysaccharide copolymerase; TPR, tetratricopeptide repeat proteins; IM, inner membrane; PG,

peptidoglycan sacculus; OM, outer membrane; c-di-GMP, bis-(3′-5′)-cyclic dimeric guanosine monophosphate; ABC transporter, ATP-binding cassette transporter.

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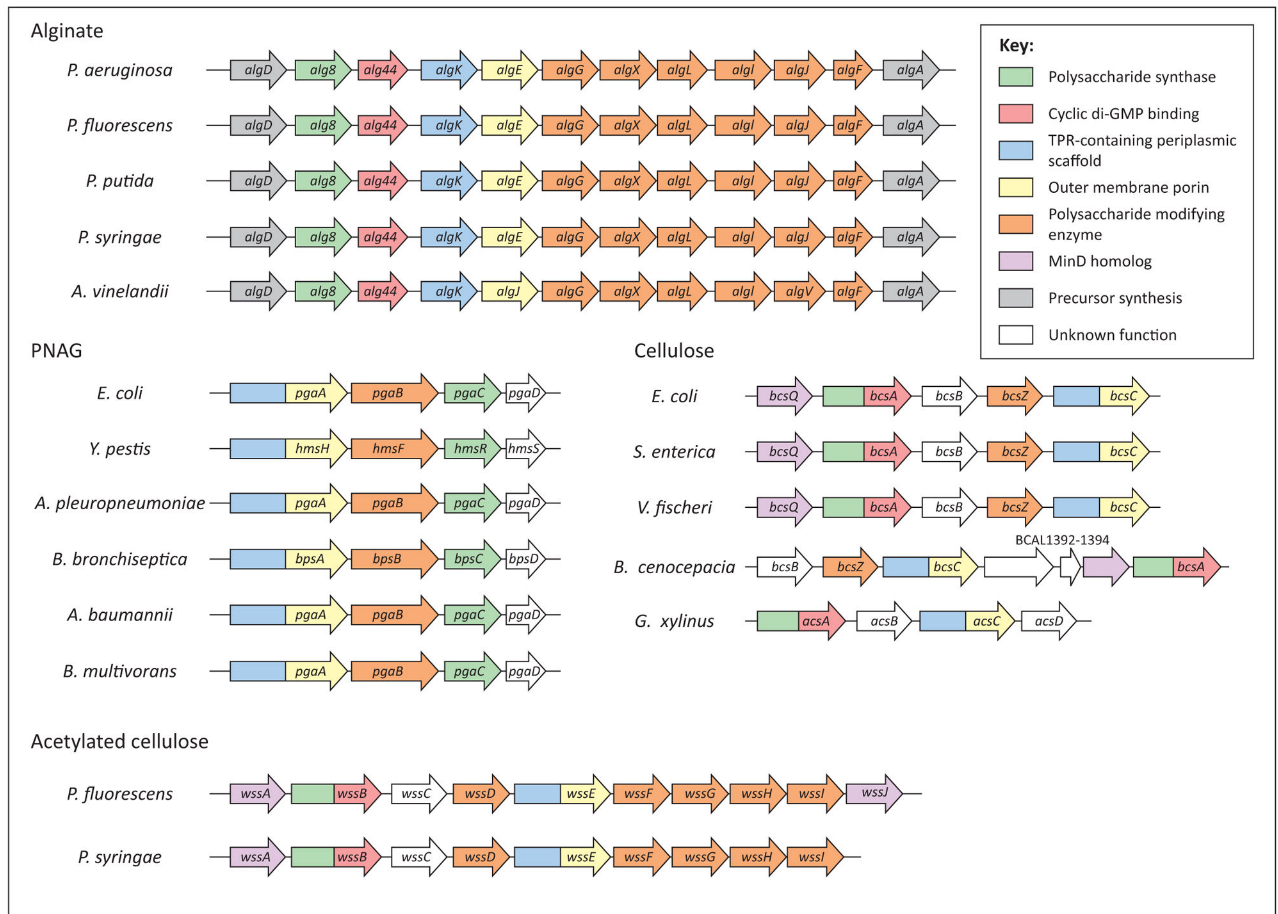

Figure 2.

Synthase-dependent exopolysaccharide loci in Gram-negative bacteria. Operons for which there is experimental confirmation of alginate, cellulose, and poly-β-D-*N*-acetylglucosamine (PNAG) production in Gram-negative bacteria. Each open reading frame is shown as an arrow (not drawn to scale) and named as labeled. The predicted or confirmed function of each open reading frame is indicated by its color as described by the legend in the figure. Open reading frames that have two colors represent two attributed functions. Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. fluorescens. Pseudomonas fluorescens*; *P. putida*, *Pseudomonas putida*; *P. syringae*, *Pseudomonas syringae*; *A. vinelandii*, *Azotobacter vinelandii*; *E. coli*, *Escherichia coli*; *Y. pestis*, *Yersinia pestis*; *A. pleuropneumoniae*, *Actinobacillus pleuropneumoniae*; *B. bronchiseptica*, *Bordetella bronchiseptica*; *A. baumannii*, *Acinetobacter baumannii*; *B. multivorans*, *Burkholderia multivorans*; *S. enterica*, *Salmonella enterica*; *V. fischeri*, *Vibrio fischeri*; *B. cenocepacia*, *Burkholderia cenocepacia*; *G. xylinus*, *Gluconacetobacter xylinus*.

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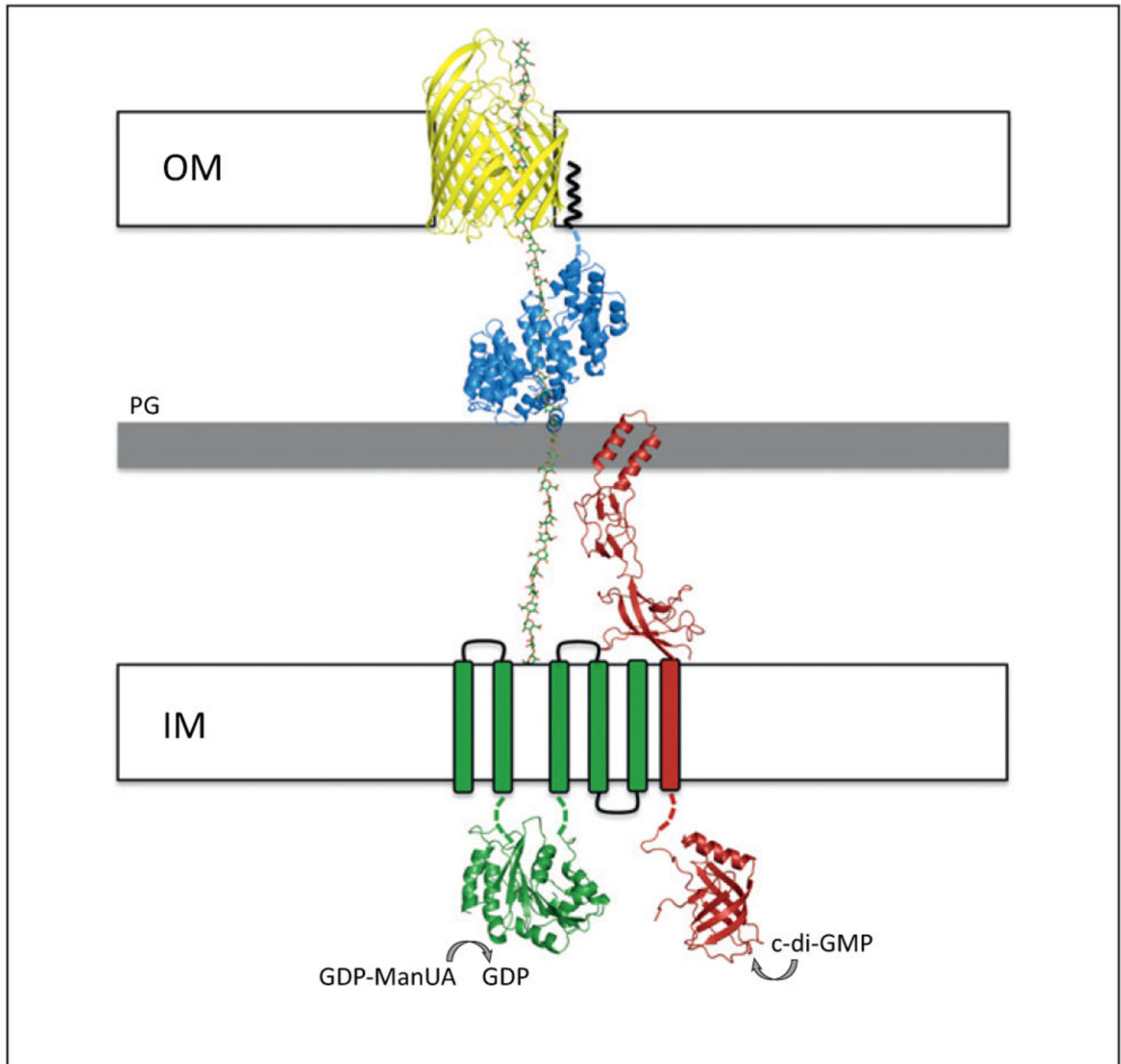

Figure 3.
Model of the polymerization and export of alginate. Structural representation of the inner- and outer-membrane components required for alginate secretion. AlgE, AlgK, Alg8, and Alg44 are displayed in cartoon representation in yellow, blue, green, and red, respectively. The AlgE (*Pseudomonas aeruginosa*) and AlgK (*Pseudomonas fluorescens*) models are derived from the recent crystal structures determined by Whitney *et al.* (PDB 3RBH) [30] and Keiski *et al.* (PDB 3E4B) [29]. The synthase domain of Alg8 was modeled using the first GT-A domain of the unpublished crystal structure of *Escherichia coli* chondroitin polymerase (PDB 2Z86). The cytoplasmic PilZ domain of Alg44 was modeled using the apo structure of *P. aeruginosa* PA4608 (PDB 1YWU) solved by the Northeast Structural Genomics Consortium [92] and the periplasmic MFP domain was modeled using *E. coli*

MacA (PDB 3FPP), the periplasmic component of a tripartite macrolide-specific efflux pump [93]. The Alg8 and Alg44 models were generated using the Protein Homology/analogY Recognition Engine (Phyre[2]) server [33]. 1,4-Linked β-D-mannuronic acid was modeled in the diagram (shown as green sticks) to demonstrate the role of AlgK as a protective scaffold protein and of AlgE as the outer-membrane alginate export protein. GDP mannuronic acid (GDP-ManUA) is the activated sugar nucleotide precursor. The black squiggly line indicates the N-terminal lipid anchor of AlgK. IM, PG, and OM refer to the inner membrane, the peptidoglycan sacculus, and the outer membrane, respectively.

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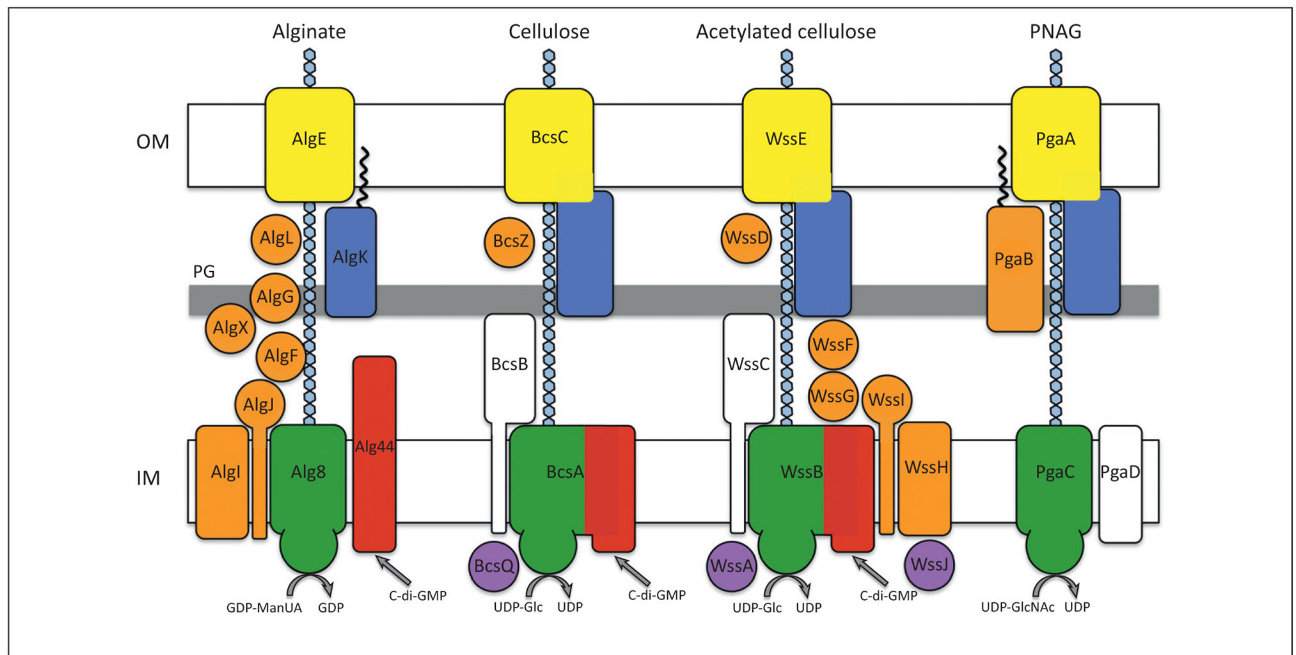

Figure 4.

Schematic representation of the alginate, cellulose, acetylated cellulose, and poly-β-D-*N*-acetylglucosamine (PNAG) exopolysaccharide secretion systems. The components for each pathway are indicated on the diagram and color-coded according to similar predicted function as follows: green, synthase; red, c-di-GMP, bis-(3′-5′)-cyclic dimeric guanosine monophosphate; blue, tetratricopeptide repeat; yellow, β-barrel porin; orange, exopolysaccharide-modifying enzyme; purple, MinD homolog; and white, unknown function. The black squiggly line indicates the N-terminal lipid anchors of AlgK and PgaB. In each system, the polysaccharide indicated is polymerized and transported across the inner membrane by its respective synthase. For alginate, cellulose, and acetylated cellulose, this process also requires the c-di-GMP receptor indicated. Once in the periplasm, various polysaccharide-modifying enzymes act on each of the polysaccharides before they are exported across the outer membrane by a tetratricopeptide repeat (TPR)-containing protein and an integral outer-membrane β-barrel. Abbreviations: IM, inner membrane; PG, peptidoglycan sacculus; OM, outer membrane; GDP-ManUA, GDP mannuronic acid; UDP-Glc, UDP glucose; UDP-GlcNAc, UDP *N*-acetylglucosamine.

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Table 1

Proteins involved in alginate, cellulose, and PNAG polymer biosynthesis and export

Protein	Predicted or demonstrated function	Subcellular localization	PDB ID[a]	Refs
Alginate				
Alg8	Synthase	Inner membrane	N/A	[41,42]
Alg44	c-Di-GMP receptor	Inner membrane	N/A	[22,41]
AlgK	TPR scaffold	Outer membrane	3E4B	[24,25]
AlgE	β-Barrel porin	Outer membrane	3RBH	[26]
AlgG	C5 mannuronan epimerase	Periplasm	N/A	[35,36,43]
AlgX	*O*-Acetylation?	Periplasm	Forthcoming	[48,49]
AlgL	Alginate lyase	Periplasm	Forthcoming	[50,51]
AlgI	*O*-Acetylation	Inner membrane	N/A	[45–47]
AlgJ	*O*-Acetylation	Inner membrane	N/A	[45–47]
AlgF	*O*-Acetylation	Periplasm	N/A	[44,46]
Cellulose				
BcsQ/WssA	Polar localization of apparatus?	Cytoplasm	N/A	[61]
BcsA/WssB	Synthase/c-di-GMP receptor	Inner membrane	N/A	[23,53]
BcsB/WssC	Unknown	Inner membrane	N/A	[59]
BcsZ/WssD	Glycosyl hydrolase	Periplasm	3QXF, 3QXQ	[60]
BcsC/WssE	TPR scaffold/β-barrel porin	Outer membrane	N/A	[25,26,59]
WssF	*O*-Acetylation?	Periplasm	N/A	[56]
WssG	*O*-Acetylation?	Periplasm	N/A	[47,56]
WssH	*O*-Acetylation?	Inner membrane	N/A	[47,56]
WssI	*O*-Acetylation?	Inner membrane	N/A	[47,56]
WssJ	Unknown	Cytoplasm	N/A	[56]
AcsD	Cellulose fiber formation	Extracellular	3AJ1, 3A8E	[62,63]
PNAG				
PgaA	TPR scaffold/β-barrel porin	Outer membrane	N/A	[65,69]
PgaB	De-*N*-acetylase	Outer membrane	4F9D, 4F9J	[65,69,71]
PgaC	Synthase	Inner membrane	N/A	[65,69]
PgaD	c-Di-GMP binding?	Inner membrane	N/A	[65,69]

[a]N/A, structure not available in PDB.