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Insights into the structure and function of membrane-integrated processive glycosyltransferases

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Abstract

Complex carbohydrates perform essential functions in life, including energy storage, cell signaling, protein targeting, quality control, as well as supporting cell structure and stability. Extracellular polysaccharides (EPS) represent mainly structural polymers and are found in essentially all kingdoms of life. For example, EPS are important biofilm and capsule components in bacteria, represent major constituents in cell walls of fungi, algae, arthropods and plants, and modulate the extracellular matrix in vertebrates. Different mechanisms evolved by which EPS are synthesized. Here, we review the structures and functions of membrane-integrated processive glycosyltransferases (GTs) implicated in the synthesis and secretion of chitin, alginate, hyaluronan and poly-N-acetylglucosamine (PNAG).

Introduction

Different mechanisms evolved to polymerize sugar molecules into high molecular weight polysaccharides. Polymer assembly by a GT requires the activation of the monomeric sugar units, often as nucleotide-bound form, and the transfer of these ‘donor’ sugars to an acceptor, a specific hydroxyl group of the growing polysaccharide chain [1••].

GTs come in many forms with finely tuned specificities for different donors and acceptors [1••]. Most GTs catalyze only a single transfer after which the enzyme-product complex dissociates. However, some GTs are highly processive enzymes that do not release the polymer product, thereby achieving astonishing polymerization efficiencies with thousands of sugar units per polymer.

Here we focus on current insights into the mechanisms of chitin, hyaluronan (HA), PNAG and alginate biosyntheses by processive GTs. Cellulose biosynthesis in plants and bacteria has recently been reviewed [2,3] and we use insights gained from bacterial cellulose synthase [4••,5••] to highlight differences and commonalities among processive GTs.

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Conflict of interest statement

The authors declare no conflict of interest.

Processive GTs form linear high molecular weight polymers

The GTs discussed below share several traits, Figure 1. The enzymes belong to family-2 of GTs [6], are membrane-integrated, and share a common cytosolic GT domain for donor and acceptor binding [1••]. These GTs transfer sugars from cytosolic nucleotide-activated sugars and generate nucleoside diphosphates (mostly UDP or GDP) as second reaction product [1••], which often competitively inhibit the synthase at elevated concentrations [7•,8•]. Glycosyl transfer is believed to occur via an S_N2-like nucleophilic displacement reaction in which the acceptor attacks the donor's anomeric C1 carbon, thereby inverting its configuration from α to β [1••]. Coupled to polymer synthesis, the enzymes translocate the nascent polysaccharide across the plasma membrane through a pore formed by their transmembrane (TM) region [4••,9–11].

The GT domain contains several sequence motifs that are required for donor and acceptor binding (reviewed in detail in Ref. [3]). Three variably spaced aspartates are crucial for catalytic activity, Figure 2 [12]. The first Asp, frequently belonging to a 'DDG' motif (text box 1 in Figure 2), contributes to nucleotide binding [4••]. The second Asp, located in the consensus 'DxD' motif (text box 2 in Figure 2), coordinates a Mg^{2+} or Mn^{2+} required for GT activity [4••]. The third Asp is also part of a tripeptide motif ('TED' in cellulose and 'GDD' in HA synthase, text box 3 in Figure 2) and probably functions as the general base that facilitates acceptor deprotonation during glycosyl transfer [4••,5••]. A fourth sequence motif particularly characteristic of processive GTs is a 'Q/ LxxRW' pentapeptide (text box 4 in Figure 2) [13•]. On the basis of the cellulose synthase structure, the Trp residue forms vander-Waals contacts with the polymer's acceptor glucose unit while the preceding Arg residue contacts the substrate's pyrophosphate group [5••].

Linear polysaccharides carry reducing and non-reducing ends, referring to the termini with an unmodified and linked hydroxyl group at the anomeric carbon, respectively. All processive GT-2 enzymes characterized to date synthesize linear polymers, Figure 3, which can be modified by soluble proteins after translocation across the plasma membrane. With only a few known exceptions, the enzymes form homo-polysaccharides in which all sugar units are connected by the same glycosidic bond.

Chitin synthase

Chitin is one of the most abundant biopolymers and occurs in various contexts across a broad range of species. It is a linear, β-1,4-linked polymer of N-acetylglucosamine (NAG), Figure 3, that is best known for its strengthening and protective role in the body wall cuticles of arthropods and several other invertebrates.

Insect chitin synthases (CHSs) have recently been reviewed in detail by Merzendorfer [9]. The enzymes can be divided into three distinguishable domains termed A, B and C. Domain A is located at the N-terminus and has limited sequence conservation among different species. In fungal class I–III and VI CHSs, the A domains do not contain any transmembrane helices (TMHs), whereas class IV + V and VII enzymes contain 2–3 TMHs [14]. The B domain forms a central cytosolic GT domain that is followed by the C domain containing 3–7 TMHs [9].

Substrate, chain elongation and primer hypothesis

Biochemical data on CHSs and related GTs, including the bacterial lipochito-oligosaccharide synthase NodC [12], led to a general reaction scheme for chitin biosynthesis. The polymer is formed from UDP-activated NAG and is extended at its non-reducing end [15,16]. Similar to the arrangement of glucose units in cellulose, the individual NAG units of chitin are rotated by approximately 180° relative to their neighbors [17]. Initial mechanistic models thus assumed that CHS binds its substrate in alternating orientations [18], resembling discussions on cellulose biosynthesis [19]. Recent insights into cellulose biosynthesis, however, explained that this alternating arrangement can be established by a simple rotation of the terminal sugar unit around the glycosidic bond, eliminating the need for a dual substrate binding site [4••].

Chitin biosynthesis has primarily been studied in *Saccharomyces cerevisiae*, which differentially expresses three *chS* genes [20]. CHS1 is responsible for only 10% of the *in vivo* chitin pool, but accounts for most of the chitin synthase activity recovered *in vitro* [21]. Interestingly, this activity is enhanced in the presence of NAG as well as chitooligosaccharides, which may prime chitin biosynthesis [22].

Regulation of chitin biosynthesis

Chitin biosynthesis is regulated on multiple levels. Regulation can directly affect CHS, its cellular localization, or the activities of enzymes involved in substrate biosynthesis [18].

The intracellular localization and stability of CHS can be controlled by phosphorylation. *S. cerevisiae* CHS2 synthesizes chitin for the primary septum [23] and is phosphorylated by Cdk1 at four positions near its N-terminus. Phosphorylation leads to the ER retention of CHS2 until mitotic kinases are degraded during cell cycle progression [24–26]. As observed for some fungal and insect CHSs, chitin biosynthesis may be stimulated by endopeptidases, such as trypsin [25]. This observation led to the suggestion that CHS enzymes exist in two states, either zymogenic or active. Yet, the biological significance of these observations is still unclear [18].

HA synthase

HA is ubiquitously expressed among vertebrates and is one of the most abundant glucosaminoglycans in the human body where it represents a major component of the extracellular matrix [27•,28•]. HA is particularly enriched in soft connective tissues aiding in osmo-regulation, cell differentiation, and cell adhesion and migration, among other functions [27•]. It is a linear polysaccharide of strictly alternating NAG and glucuronic acid (GA) units linked via β-1,3 and β-1,4 glycosidic linkages, respectively, Figure 3. The polymer can reach astonishing lengths, ranging from several hundred to tens of thousand disaccharide units. Importantly, the physiological effects of HA are modulated by its length. It has recently been reviewed [27•,29] that low and high molecular weight HA exerts opposing physiological effects, including pro or anti-inflammatory and angiogenic properties. However, it is also possible that low molecular weight HA is a consequence, rather than the cause, of inflammatory processes as it can be chemically degraded by, for example, reactive oxygen species [30].

HA is also produced by some bacteria and even viruses [31,32•]. The identification of bacterial hyaluronan synthases (HAS) by Weigel and colleagues paved the way for a detailed biochemical characterization [33,34,35••]. While bacterial HA (primarily produced by vertebrate pathogens) forms a non-immunogenic capsule component probably to avoid host immune responses, the biological significance of viral HA is currently unclear. Although most bacterial HASs are membrane-integrated enzymes like their eukaryotic counterparts, a second class of bacterial HAS has been identified in *Pasteurella multocida*, that is non-processive and not membrane-integrated [36].

HAS are small membrane-integrated GTs

Compared to cellulose, chitin and alginate synthases, HASs are remarkable enzymes with unique properties. First, HAS recognizes two different substrates, UDP-NAG and UDP-GA; second, the enzyme catalysis the formation of β-1,3 and β-1,4 linkages; third, HA has a strictly alternating NAG-GA sequence; and fourth, HAS is necessary and sufficient to catalyze the synthesis and membrane translocation of HA [10••].

Bacterial and vertebrate HAS differ with respect to their predicted TM topologies, containing 4–5 and 6–7 TM regions, respectively. The enzymes share a conserved cytosolic GT domain, usually located between TMHs 2 and 3. Radiation inactivation analyses on *Streptococcal* HAS showed that the enzyme functions as a monomer [34], however, recent immuno-precipitation analyses on human HAS suggest homo-oligomerization of the enzyme [36].

Studies on bacterial HAS provided the first biochemical evidence for the dual functionality of processive GTs. Hubbard and colleagues reconstituted purified *Streptococcal* HAS into proteoliposomes for HA translocation studies [10••]. By initiating HA biosynthesis with substrates added to the outside of the lipid vesicles, this study demonstrated that the newly synthesized HA indeed accumulated in the vesicle lumen, thereby corroborating that bacterial HAS is necessary and sufficient for the synthesis and translocation of HA [10••,11].

In contrast to most other processive family-2 GTs characterized to date, HAS appears to elongate the reducing end of HA. This was demonstrated using primarily pulse-chase labeling of HA produced by bacterial and vertebrate enzymes and requires that the growing HA polymer is attached to UDP [37–41]. Hence, UDP-activated GA and NAG would function as the acceptors during glycosyl transfer and not as donors [10••]. Further structural and functional analyses are required to corroborate this mechanism and to delineate architectural adaptations of HAS that distinguish it from other processive family-2 GTs.

Regulation of HAS activity

Human HAS isoforms 1, 2 and 3 have been reported to produce HA polymers of different molecular weight [42], while evidence for length regulation of bacterial HA is sparse. Transcriptional control of *haS* genes and the availability of substrates might be one mechanism by which HA polymerization is controlled [43]. The biosyntheses of UDP-activated GA and NAG depend on the cytosolic glucose concentration as well as the regulation of enzymes required for their biosyntheses. For example, over-expression of *S.*

equisimilis HAS together with UDP-glucose 6-dehygrogenase, glucose-1-P uridyltransferase, and NAG-uridyltransferase in *E. coli* led to the accumulation of high titers of HA in the growth medium [44]. In addition, Kumari and colleagues identified two conserved polar amino acids in *Streptococcal* HAS TMH2 and TMH4 that are crucial for the biosynthesis of high MW HA [45] and Pummill and DeAngelis identified Ser77 in *Xenopus* HAS1 as a crucial residue for HA length regulation [46]. HAS1 mutants carrying a point mutation at position 77 either produced lower or higher MW HA, depending on the amino acid introduced.

Posttranslational modifications

In vivo and *vitro* studies on vertebrate HAS have shown that the enzymes can be post-translationally phosphorylated, glycosylated and ubiquitinated [47–49]. For example, human HAS2 is glycosylated on Ser221 with NAG (O-GlcNAc), which increases the enzyme's activity and stability [50]. Human HAS3 expressed in COS-7 cells is phosphorylated at one or more serine positions with unknown impact on enzymatic function [49]. In another study, mouse HAS2, which is essential for normal fetal development, was shown to be mono-ubiquitinated on Lys190 [48]. Replacing Lys190 with Arg abolished the *in vitro* activity of the enzyme. Lys190 belongs to a 'GKR' motif of the GT domain that is conserved among bacterial and eukaryotic HAS, supporting its putative regulatory role, Figure 2.

Alginate synthase

Alginate is a major component of the cell wall in brown algae but is also produced by some bacteria as a biofilm component. In particular, alginate production by *Pseudomonas aeruginosa* in cystic fibrosis patients correlates with poor prognosis and increased morbidity.

Alginate is a hetero-polysaccharide of β-1,4 linked D-mannuronic acid interspersed with L-guluronic acid units [51], Figure 3. Guluronic acid is the C5 epimer of mannuronic acid. In bacteria and algae, it is introduced into the polymer by epimerization of mannuronic acid units by a soluble epimerase after the secretion of the polymer across the plasma membrane [52,53]. The distribution of these two units in the polymer varies between species [54], but usually consists of homogenic regions of the same sugar joined by heterogenic regions. The polymer can further be acetylated in the periplasm on the mannuronic acid's C2 and C3 hydroxyl groups [55].

Alginate biosynthesis in gram-negative bacteria requires a multi-component complex that probably spans the inner and outer membrane (recently reviewed by Whitney and Howell [56]). At the inner membrane, a complex of the GT Alg8 and the non-catalytic Alg44 subunits forms the active synthase that binds the substrate GDP-mannuronic acid and appears to be sufficient for alginate synthesis and membrane translocation [57•]. Transport across the outer membrane is achieved by a complex of the outer membrane porin AlgE and periplasmic AlgK subunits [58]. It is currently unknown whether the Alg8/Alg44 complex directly interacts with the outer membrane AlgK/E components [59]. An attractive model would be that alginate interacts with an ensemble of modifying enzymes within the

periplasm and that these subunits chaperone the polymer from the inner to the outer membrane [56,60].

Regulation of alginate biosynthesis

Alg8 contains 5 predicted TMHs with the GT domain between TMH2 and 3 [57•]. The subunit is catalytically inactive in the absence of Alg44 [61], which contains a single TMH framed by an intracellular N-terminal PilZ domain and a periplasmic C-terminal domain. PilZ domains, first identified as regulatory components of cell motility, bind the bacterial signaling molecule cyclic-di-GMP, which affects many cellular processes, including biofilm formation [62•]. Similar to bacterial alginate production, synthesis of bacterial cellulose is allosterically activated by cyclic-di-GMP [7•,63••]. Here, the activator binds to a PilZ domain located at the C terminus of the catalytic cellulose synthase subunit BcsA. Cyclic-di-GMP binding affects the accessibility of BcsA's active site via a 'gating loop' [3,4••,5••]. How cyclic-di-GMP regulates alginate biosynthesis is currently unknown but is particularly important for the development of novel antimicrobial therapeutics.

PNAG synthase

Poly-NAG (PNAG) is an extracellular homo-polysaccharide frequently found in bacterial biofilms [64,65]. It is produced by the Pga machinery consisting of the PgaABCD proteins [66,67•] and is formed from UDP-activated NAG that is polymerized via β-1,6 linkages, Figure 3.

Similar to bacterial alginate and cellulose synthases, the Pga complex probably spans the inner and outer bacterial membrane [56]. PgaC and PgaD form an inner membrane-integrated complex. PgaC is the catalytic GT containing 4–5 predicted TMHs. PgaD probably contains two TMHs with a short periplasmic loop and small cytosolic C terminus. PgaA is an outer membrane porin similar to AlgE [58] and might associate with PgaB, a periplasmic lipoprotein that is probably anchored to the outer membrane. PgaB deacetylates a small fraction of the polymer's NAG units [68].

Production of PNAG strongly depends on the presence of cyclic-di-GMP, similar to alginate and cellulose biosynthesis [69••]; however, PgaC or D lack classical cyclic-di-GMP-binding domains [62•]. With a clever cross-linking and mutagenesis approach, Steiner and colleagues demonstrated that cyclic-di-GMP interacts with and stabilizes a PgaCD complex [69••]. In the absence of cyclic-di-GMP, PgaCD dissociates, rendering PgaD prone to proteolysis. It is thus likely that allosteric activation of PNAG biosynthesis by cyclic-di-GMP is based on stabilization of the PgaCD interaction [5••].

BcsA as a model for processive GTs?

Biochemical and structural analyses suggest that processive family-2 GTs directly couple polysaccharide synthesis and secretion, Figure 1. Structural analyses on bacterial cellulose synthase revealed that this is achieved by a tight association of the GT-domain with pore forming TMHs [3]. The cellulose synthase structure demonstrates that the binding site for the acceptor (i.e. the cellulose's non-reducing terminal glucose unit) is formed directly at the

entrance to the TM-channel, such that the elongated polysaccharide translocates into the TM pore rather than dissociates from the enzyme [5••], Figure 4. The interface between the GT domain and TM channel is formed by amphipathic interface helices, one of which contains the Q/ LxxRW motif implicated in acceptor binding [3].

A sequence comparison of cellulose, chitin, HA, alginate and PNAG synthases is difficult based on primary sequences alone. However, considering the sequence conservation of the GT domains, predicted TM topologies and secondary structures, as well as experimental topology studies [57•,70], we aligned the sequences of selected GT-2 family members, Figure 2.

Interestingly, TMH predictions [70] of the region directly C-terminal of the GT domains of HA, alginate and chitin synthases predict either a long TMH or two TMHs linked via a tight 2–3 residue long extracellular loop. This region corresponds to TMH5 in BcsA [4••], which forms a cytosolic interface helix over its N terminal half and inserts into the membrane past a conserved Pro residue. The amphipathic portion of the TMH is part of the TM channel entrance. Thus, it is possible that a curved TMH is a common feature of processive GTs. The sequence alignment shown in Figure 2 can only be a cautious attempt to compare BcsA with other processive enzymes but should aid in the design of biochemical experiments to further characterize these exceedingly important enzymes.

The limited space available precluded discussing many other polysaccharides formed by processive GTs, including bacterial acetylated cellulose, mixed linkage glucans in plants and bacteria, as well as curdlan.

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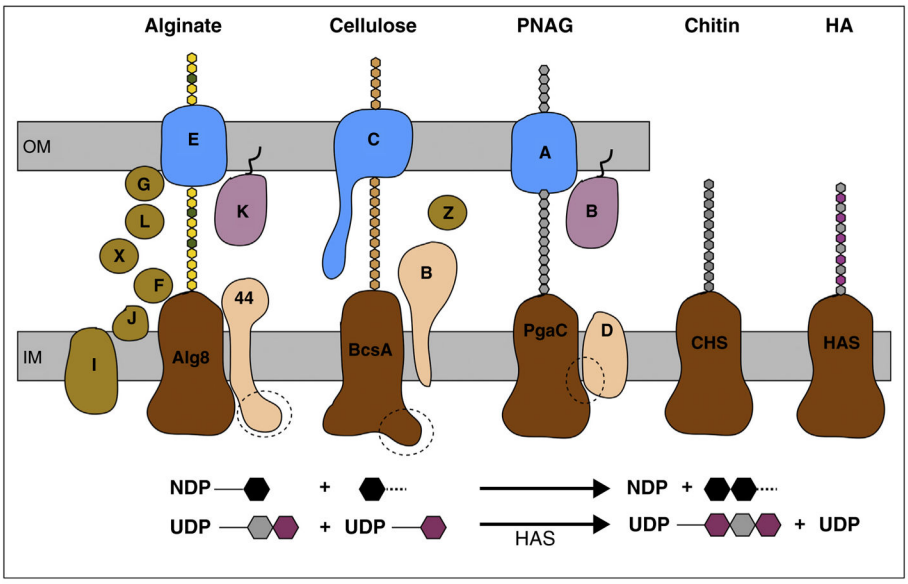

Figure 1.

Membrane-integrated processive GTs synthesize and secrete diverse polysaccharides. The synthases may be part of multi-component complexes or function on their own. The catalytically active subunits (colored brown) share an intracellular GT and a membrane-integrated domain. Alginate consists of mannuronic (yellow) and guluronic acid (green), cellulose of glucose (beige), PNAG of NAG (gray), chitin of NAG, and HA of NAG and GA (magenta) units. A dashed circle indicates the binding site for the signaling molecule cyclic-di-GMP. Lower panel: The enzymes catalyze the transfer of a nucleotide diphosphate (NDP)-activated sugar (black hexagon) to another glycosyl unit, thereby generate NDP as a second reaction product. Among the synthases shown, HAS is the only enzyme that appears to elongate the polymer at its reducing end, thereby generating an UDP-attached polysaccharide. OM, IM: Outer and inner membrane.

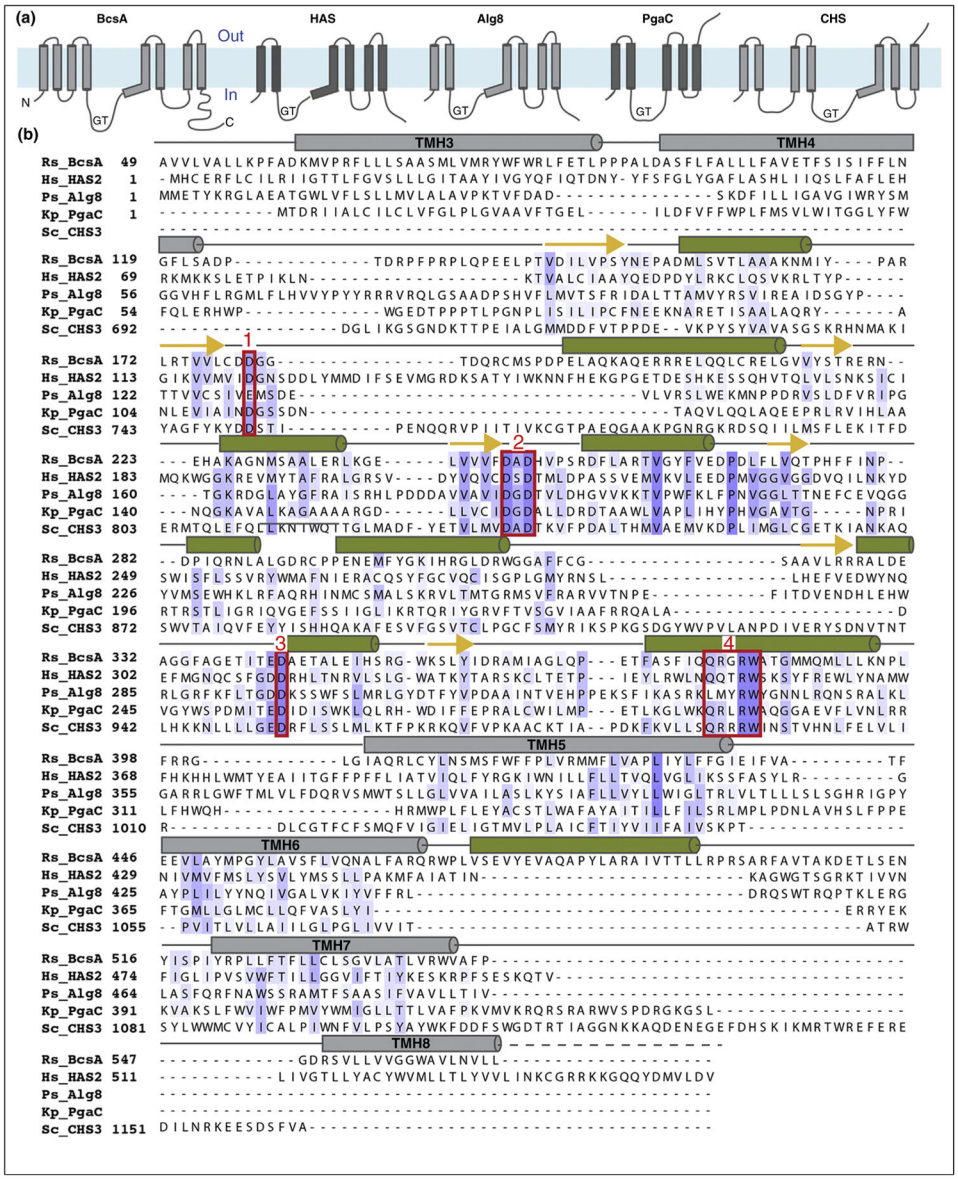

Figure 2.

Sequence alignment and predicted secondary structure of selected family-2 GTs. **(a)** Predicted TM topology of *Rhodobacter sphaeroides* BcsA, *Homo sapiens* HAS2, *Pseudomonas aeruginosa* Alg8, *Klebsiella pneumoniae* PgaC, and *Saccharomyces cerevisiae* CHS3. Topology diagrams are shown from the N to the C terminus, labeled N and C for BcsA. GT: glycosyltransferase domain. The membrane region is shown as a blue rectangle. Topologies were predicted with TOPCONS [71]. **(b)** Multiple sequence alignment of the sequences used in (a). The sequences were aligned in CLUSTALW [72] and predicted TM topologies and secondary structures were used to manually refine the alignment. Predicted TM helices and secondary structure elements are shown as gray bars (TM helices) and green columns and yellow arrows for α-helices and β-strands, respectively. Conserved sequence motifs are framed with a red box and numbered 1–4.

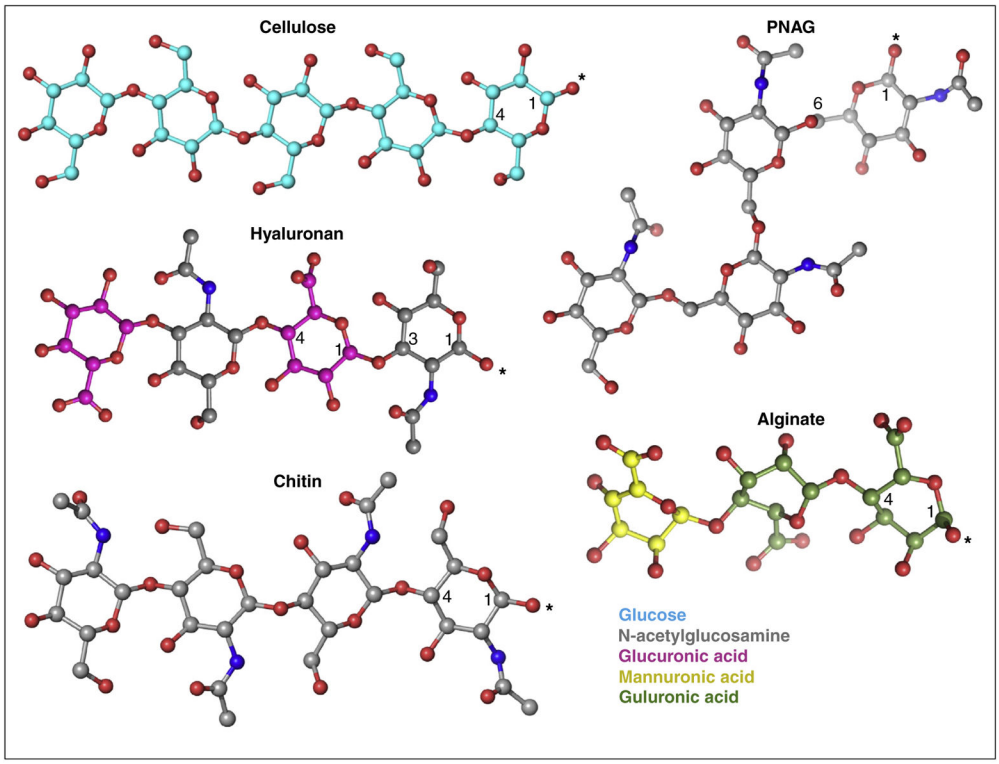

Figure 3.

Chemical diversity of polysaccharides. Coordinates for the shown oligosaccharides were obtained and adjusted from pdb entries 4P02 (cellulose), 3AFL (alginate), 2JCQ (HA), 4P7R (PNAG), and 3WH1 (chitin). The carbon atoms of the individual sugars are colored as in Figure 1, oxygen and nitrogen atoms are colored red and blue, respectively. The polymer's reducing ends are labeled with an asterisk.

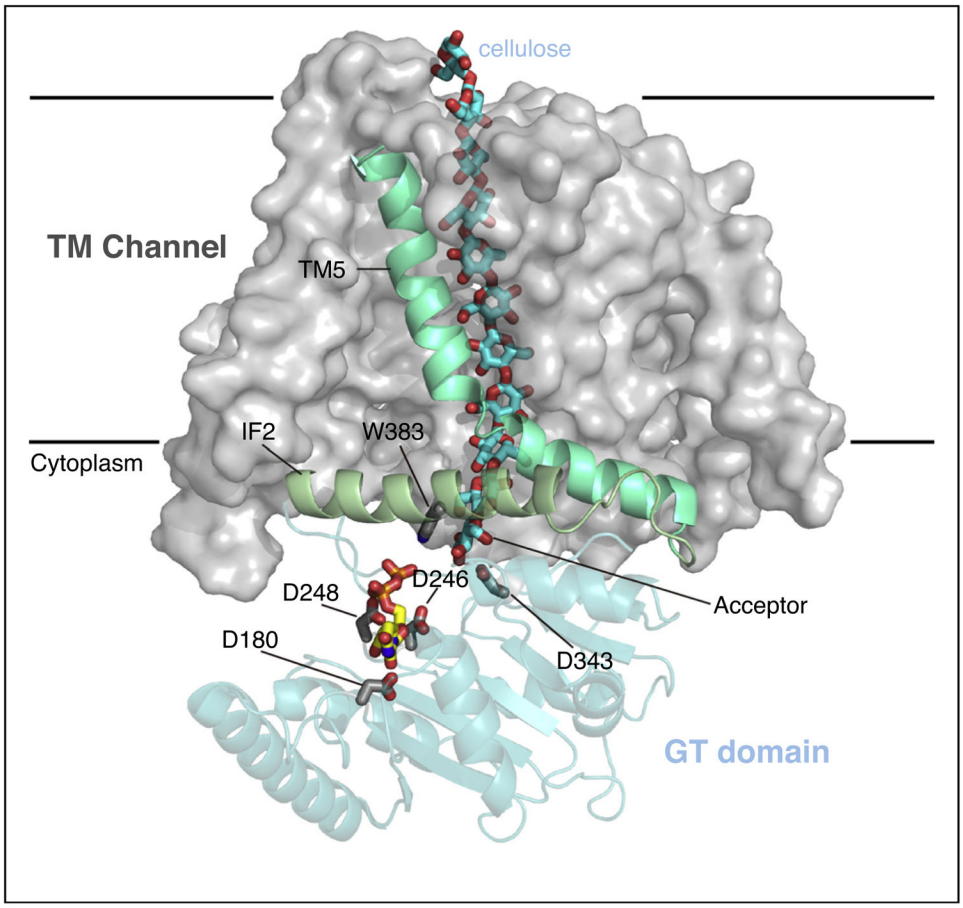

Figure 4.

Pore organization of bacterial cellulose synthase subunit BcsA. BcsA's TMHs are shown as a gray surface with the exception of TMH5 and IF2, which contains the 'QxxRW' motif. The GT domain is shown as a blue cartoon and cellulose and UDP are shown as sticks with blue and yellow carbon atoms, respectively. Selected residues belonging to the conserved DDG (D180), DxD (D246 and 248), TED (D343), and Q/ LxxRW (W383) motifs are shown as sticks. Cellulose synthase elongates the polymer at the non-reducing end, which is stabilized at the acceptor site within the catalytic pocket.