Controlled processivity in glycosyltransferases: a way to expand the enzymatic toolbox

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Abstract:

Glycosyltransferases (GT) catalyse the biosynthesis of complex carbohydrates which are the most abundant group of molecules in nature. They are involved in several key mechanisms such as cell signalling, biofilm formation, host immune system invasion or cell structure and this in both prokaryotic and eukaryotic cells. As a result, research towards complete enzyme mechanisms is valuable to understand and elucidate specific structure-function relationships in this group of molecules. In a next step this knowledge could be used in GT protein engineering, not only for rational drug design but also for multiple biotechnological production processes, such as the biosynthesis of hyaluronan, cellooligosaccharides or chitooligosaccharides.

Generation of these poly- and/or oligosaccharides is possible due to a common feature of several of these GTs: processivity. Enzymatic processivity has the ability to hold on to the growing polymer chain and some of these GTs can even control the number of glycosyl transfers. In a first part, recent advances in understanding the mechanism of various processive enzymes are discussed. To this end, an overview is given of possible engineering strategies for the purpose of new industrial and fundamental applications. In the second part of this review, we focused on specific chain length-controlling mechanisms, i.e. key residues or conserved regions, and this for both eukaryotic and prokaryotic enzymes.

1. Introduction

Glycosyltransferases (GTs) form natural glycosidic linkages by catalysing the transfer of an activated sugar donor onto saccharide and non-saccharide acceptors. GTs are classified (EC.2.4x.y.) based on sequence similarities, e.g. 3D-structure or chemical reaction mechanism, and collected in the Carbohydrate Active Enzyme database (CAZy, http://www.cazy.org/) (Lombard et al., 2014). In contrast to this simple classification stand the complex and vital processes that glycosylated molecules or glycosidic-linked molecules, i.e., glycans, are involved in. In particular this is true for the carbohydrates, the most abundant group of biomolecules in nature as they are predominantly regarded as energy sources, the backbones of nucleic acids and major cell wall constituents with a broad (patho)physiological significance. More recently, the importance of carbohydrates to carry molecular information and versatile signals for the cell's communication is being recognised (Cocinero and Carçabal, 2015; Gabius, 2018, 2000). In addition, glycosylated molecules are present in all branches of the evolutionary tree (Corfield and Berry, 2015). In this regard, after nucleic acids and proteins, glycans can be seen as a third class of structural variable and flexible molecules with the ability to store biological information (Cocinero and Carçabal, 2015; Gabius, 2018, 2000). However, elucidating the structure-function relationships of this group of molecules is severely hindered due to the fact that current production technologies are not able to produce these glycans in a selective, scalable and economical way (Danishefsky et al., 1993; Wong, 2015; Yamada et al., 1999; Zhang et al., 1999).

In nature, the complex and structural diversity of carbohydrates is made possible by the collaboration of many different enzymes: the GTs. Therefore, this natural process can be applied to several biotechnological production processes, using these native or engineered GTs, and thereby circumventing many of the disadvantages of chemical synthesis. In the context of using GTs for the biotechnological production of complex glycans, several metabolic engineering strategies (Endo et al., 1999; Kim et al., 2015; Liu et al., 2008; Malla et al., 2013; Yoshimura et al., 2015) have been used to augment the in vivo nucleotide (sugar) pool for the biosynthesis of numerous glycans, ranging from the glycoside quercitin 3-O-NAG (Kim et al., 2012) and the oligosaccharide lacto-*N*-tetraose (Baumgärtner et al., 2014), to the polysaccharides hyaluronic acid (Woo et al., 2019) and heparosan (Restaino et al., 2013). Next to optimising the nucleotide (sugar) pool of these engineered microbes, a specific interest and opportunity lies in GT protein engineering and more specifically engineering of GTs which can perform iterative glycosylations with no release or purification of a product intermediate: the processive GTs. They hold on to the growing end of the carbohydrate chain making additional glycosylation rounds possible (Fig. 1a). Multiple interesting processive GTs belong to family 2 (GT2s), e.g. cellulose, chitin, hyaluronan or glucan synthases. Full insight in the mechanism of processivity and control of chain length could open the way towards understanding and intervening with other β-glycosyltransferases, considering the prominent role of these specific GTs for several biological functions. As β-glycosyltransferases are often membrane-associated proteins, they play an important role in cell signalling, motility, proliferation, cell structure, etc. and this in both prokaryotic and eukaryotic cells. In this class of β-glycosyltransferases, both high- (degree of polymerisation (DP) > 6000 monomers, polysaccharides) and low-molecular weight (DP < 20 monomers, oligosaccharides) saccharides are synthesised. It is therefore of particular interest to understand the different binding strategies for several processive GTs and how a discrepancy is made between the production of poly- or oligosaccharides (Chhetri et al., 2020). Only a few articles in literature already explored the possibilities to engineer and/or alter the final carbohydrate chain-length. Nonetheless, a plethora of

valuable research was conducted to (partly) elucidate the structural and functional mechanisms behind processive GTs. To understand the true mechanism of length control in biopolymers, a clear distinction between a distributive and a processive mechanism needs to be made (Breyer and Matthews, 2001). In a distributive mechanism, the enzyme releases the product after addition of the monomer which means that only long product length is formed after a longer amount of time (Poisson distribution) (Fig. 1c). In contrast, a processive mechanism retains the product during multiple rounds of elongation.

Even more interesting - and still underexplored - are the semi-processive, length-controlling GTs which often have an unknown counting mechanism that catalyses the reaction of a limited number of glycosyl transfers, before they release the product (Fig. 1b). The possibility to fully control (semi-)processive GTs and in this way even alter chain length of biologically important molecules, offers several opportunities in industrial biotechnology but also for fundamental research, e.g. understanding important structure-function relationships. Hence, in this literature review, an overview is given of both processive and semi-processive GTs complemented with recent and suggested protein engineering efforts to understand and change chain length. Recent reviews focus on the methods to evaluate processivity (Yakovlieva and Walvoort, 2019), whereas here we focus on the structural and mechanistic features associated with processivity.

2. To be processive or not to be processive?

Although carbohydrate polymers are the most abundant molecules in nature, mechanisms behind chain length control are not fully understood. This is in contrast to other biological polymers, e.g. nucleic acids and proteins, for which the polymerisation mechanism is clarified (Berthelot et al., 2004; Mirande, 2010; Uyemura et al., 1978; Wu et al., 2017). As the length of homo- and heteropolymers influences their physiological role, it is important to understand the nature of a processive versus a semi-processive reaction mechanism. This knowledge can be used for several engineering purposes, e.g. use of chain controlling enzymes in industrial applications, or understanding how this chain length affects biological functions, as carbohydrate polymers can have a prominent role in several pathogeneses, and hence results in an unique opportunity for species-targeted drug development. Even for the most abundant organic polymer on earth, cellulose, it has been less than a decade that we understand how cellulose synthase is able to catalyse the polymerisation of more than 10 000 monomers and still not everything has been discovered. For example, it remains a hypothesis how initiation of the glycan chain occurs let alone termination of synthesis. The same can be said for chitin, an important component of the cell wall of many fungi, insects and crustacea. However, recent work on for example hyaluronan synthase, shows a promising trend towards understanding mechanisms behind substrate recognition, priming and subsequent initiation of the glycosyltransferase activity (Maloney et al., 2022).

Next to the assembly of thousands of these residues, there exist GTs that can catalyse a controlled number of residues producing interesting oligosaccharides instead of polysaccharides. In contrast to polysaccharides, oligosaccharides are only made of a few number of monosaccharides. As they catalyse the addition of several residues, called ' semi-processive', it seems that they consist of an internal molecular ruler mechanism different from cellulose or chitin synthase. The processivity mechanism occurs across different GT families and although not classified together, still have similar characteristics. As shown below, recent advances and insights reveal similar features of processivity

for both eukaryotic and bacterial GTs. Focusing on the structural evidence, a general engineering strategy for chain-length control was proposed.

2.1. Structural evidence of processivity across the kingdoms of life

Cellulose and chitin synthase both belong to the family 2 of GTs (GT2s) which have an inverting reaction mechanism and adopt a GT-A fold (Dorfmueller et al., 2014; Morgan et al., 2013; Orlean and Funai, 2019; Salgado-Lugo et al., 2016). The GT-A fold differs from the GT-B fold in that both β/α/β Rossmann-like domains are abutting, as a N- and C-terminal domain, and generally metal ion dependent (Chang et al., 2011; Liu and Mushegian, 2003; Rosén et al., 2004) (Fig. 2). Since the solved crystal structure of cellulose synthase (CeS) BcsA-B (catalytic BcsA and periplasmic BcsB subunit) from *Rhodobacter sphaeroides*, a general mechanism for β-glycosyltransferases has been postulated: glycosyl transfer occurs via an S_N2-like nucleophilic displacement mechanism in which the acceptor attacks the donor's anomeric C1 carbon, thereby inverting its configuration from α to β (Lairson et al., 2008; Morgan et al., 2013).

Several studies were needed to understand how GT2s, such as cellulose or chitin synthase (Chs), were able to catalyse several glycosyl transfer reactions without releasing the intermediate product. At first, a dual active-center model explained the processivity of celullose synthase (Carpita, 2011; Charnock et al., 2001; Koyama et al., 1997; Saxena et al., 1995; Saxena and Brown, 1997). Knowledge of the BcsA structure abolished this two-domain hypothesis and revealed that GT2s consist of only one domain in the GT-A fold (Dorfmueller et al., 2014; Morgan et al., 2013). Although there are some differences between cellulose synthases (CeS) of bacteria and plants, they share a similar topology with two transmembrane (TM) helices at the Nt and at least four Ct TM helices ((Pear et al., 1996) and reviewed by (Slabaugh et al., 2014) and (McNamara et al., 2015)). Altogether, these TM helices form a pore through the membrane, extruding the nascent glycan chain (Fig. 3). Literature suggests the presence of an 18 residues long gating loop contributing to the processive cycle to produce cellulose. The activator cyclic di-GMP binds to the PilZ domain, named after the PilZ protein family which are the best studied class of c-di-GMP effectors (Ryjenkov et al., 2006), breaking a salt bridge and in this way inserting a gating loop and coordinating the nucleotide sugar, UDP-Glc, to bind in a processive cyclic manner. Next, a 12 residues long 'finger helix' interacts with the acceptor end of the cellulose chain, facilitating its translocation into the TM channel (Knott et al., 2016; Morgan et al., 2014). Importantly, the glycan chain is elongated at the non-reducing end (Koyama et al., 1997; Lai-Kee-Him et al., 2002). Sequence motifs implicated in this catalysis are discussed in Section 2.3. While we almost completely understand how the glycosyl transfer occurs for this processive β-glycosyltransferases, not all mechanisms for priming of glycan synthesis and release of the glycan chains, often associated with processivity mechanisms, are known.

Recently, several hypotheses were postulated explaining the priming and elongation of chitin chains as their length distribution is different dependent on the Chs involved and is important for cell wall biogenesis (Gyore et al., 2014; Orlean and Funai, 2019; Salgado-Lugo et al., 2016). In fungi, these chitin containing cell walls are important factors in plant-fungal interactions and the chitin composition or distribution are crucial both in defence and symbiosis of plant cells (reviewed by (Pusztahelyi, 2018)). Interestingly, it has been shown that Chs self-primes initiation by generating GlcNAc from UDP-GlcNAc. This is different from the CeS activator cyclic-di-GMP which breaks a salt bridge controlling the access towards the active site (Morgan et al., 2014). These findings implicate that Chs synthesise chitin chains in 'bursts' as polymerisation can be reinitiated once the primer GlcNAc is present. How

these different chitin chain distributions impact the cell wall construction is not fully understood but this is probably a combination of UDP-GlcNAc availability, regulatory proteins and the organisation of Chs in the membrane, e.g. ability to form parallel or anti-parallel interchain networks (Orlean and Funai, 2019; Salgado-Lugo et al., 2016) (Fig. 4). Next to the chitin synthesis model depicted by (Dorfmueller et al., 2014), the self-priming principle could further enlarge the platform for the discovery of antifungal targets. Most results for the chitin synthase mechanism are based on in vitro/in vivo enzyme studies or bioinformatic analysis (Dorfmueller et al., 2014; Gohlke et al., 2017). However, very recent work elucidated several structural insights on the chitin biosynthesis in both *Candida albicans* and *Phytophtora sojae* (Chen et al., 2022; Ren et al., 2022). Understanding why and how one or a combination of Chs produce a certain chain length distribution could shed light on Chs function and classification.

Another important constituent of the inner layer of the fungal cell wall, next to chitin, is glucan. The (1,3)-β-Glucan chain is synthesised by (1,3)-β-glucan synthase (GluS) and then further modified in the periplasmic space. Similar to cellulose synthase, GluS catalyses the sequential addition of UDP-Glc (donor) to Glc (acceptor) and this by a (1,3)-β-glycosidic linkage instead of a (1,4)-β linkage (Douglas, 2001). Next to chitin synthase, GluS is also an interesting target for the development of antifungals. GluS is a processive enzyme and fungal/plant GluS variants are classified in the GT48 family. Up until recently, it was believed that GluS produced glucan with an average DP of 60-80 (Shematek et al., 1980). However, a recent study by (Chhetri et al., 2020), has proven that GluS alone synthesised linear (1,3)-β-glucan with an average DP of 6550 ± 760. These findings indicate again the importance to understand the mechanism of processivity and which factors influence chain length. Knowing that GluS alone is responsible for full polymerisation of (1,3)-β-glucan will switch the search for antifungal targets to GluS inhibitors instead of other subsequent enzymes in the pathway. Finally, (1,3)-β-glucan polymerisation is initiated by self-priming with UDP-Glc, similar to chitin synthase, and will probably affect chain length (Chhetri et al., 2020; Orlean and Funai, 2019).

Complementary to CeS and Chs, the wall teichoic acid β-glycosyltransferase TarS is classified in the GT2 family with an inverting mechanism and GT-A fold. Elucidation of the catalytic features of these enzymes aid in the development of anti-methicillin resistant drug design. These techoic acids are present in the cell wall of Gram-positive bacteria, such as *Staphylococcus aureus* (Neuhaus and Baddiley, 2003). The latter is one of the leading examples in the battle against wide-spread β-lactam antibiotic resistance (Diekema et al., 2001). Wall teichoic acids (WTAs) consist of polyribitol phosphate (acceptor) attached to the peptidoglycan. Furthermore, the acceptor is heavily substituted with α- or β-O-linked GlcNAcs (Sanderson et al., 1962). One of the enzymes responsible for GlcNAcylation of these WTAs is the β-glycosyltransferase TarS. In this way, TarS is one of the key features in methicillin resistance by specifically decorating these WTAs with β-O-linked GlcNAc chains (Sobhanifar et al., 2016). The catalytic mechanism of TarS has two structural features: the presence of a catalytic site and substrate access loop. Upon binding of the donor UDP-GlcNAc, the former loop positions towards the catalytic center and the latter loop becomes ordered. In this way, the enzyme controls correct acceptor and donor binding (Sobhanifar et al., 2016). More interesting is the presence of a trimerisation domain composed of tandem carbohydrate binding domains and proven to play a role in TarS processivity (Sobhanifar et al., 2016). This would prevent premature release of the glycan chain and hence additional rounds of chain elongation.

In contrast to CeS and Chs, starch (StaS) and glycogen (GS) synthase belong to GT family 5 and structural information describes a predominant GT-B fold. Both enzymes are also considered to be processive as they both catalyse the transfer of glucose, to the non-reducing end, from a UDP-Glc or ADP-Glc donor to a glucose acceptor chain via α-1,4-linkages (Cuesta-Seijo et al., 2013; Díaz et al., 2011; Momma and Fujimoto, 2012; Nielsen et al., 2018). Multiple StaS exist dependent on the starch component: granule-bound starch synthase (GBSS) for amylose synthesis or soluble StaS I-V for amylopectin synthesis. The knowledge behind processivity mechanisms of these StaS is still scarce but recent crystallographic work revealed the presence of extra loops surrounding the reducing end glucose in non-plant GBSS (Baskaran et al., 2011; Nielsen et al., 2018). In the case of GS, several accessory sites with carbohydrate binding domains, distinct from the catalytic site, are involved in the processivity mechanism, enabling tight association with the product (Baskaran et al., 2011; Díaz et al., 2011; Sheng et al., 2009). For all GS, these glycogen association sites are located on the surface of the enzyme although differences in the number of sites and localisation (Nt versus Ct) exist between bacterial and eukaryotic GS (Baskaran et al., 2011). Similar to CeS, Chs and StaS, the number of elongation rounds catalysed by GS is less clear, however have an important impact on cellular function. A final example of a processive glycosyltransferase outside the GT2 family is TarM. This enzyme is classified in the GT4 family with a retaining reaction mechanism (Lombard et al., 2014; Xia et al., 2010). As discussed for TarS, TarM plays an important role in the decoration of WTAs. TarM catalyses α-O-GlcNAcylation to the acceptor polyribitol phosphate instead of β-O-linked GlcNAcs by TarS. Similar to TarS, (Sobhanifar et al., 2015) have demonstrated the processivity of TarM. However in TarM, not the trimerisation domain but a positively charged groove facilitates binding of the negatively charged WTA acceptor again preventing premature release of the product. In addition, they postulate that the transition from an open to closed state, a feature that is already detected in several processive glycosltransferases, would create a motion along the acceptor binding groove which would further aid to enzyme processivity (Sobhanifar et al., 2015). Although TarM and TarS are crucial targets for rational drug design, the importance of environmental influence in α- and β-glycosylation of WTAs also needs to be considered. A significant glycosylation diversity of WTAs was observed among different *S. aureus* strains, an insight that influences the decision to target TarM or TarS for inhibitor screening (Gerlach et al., 2018; Mistretta et al., 2019).

The ability of these processive enzymes to prolong association with their end-product can have an important impact on cellular function, e.g. chitin size distribution affecting cell wall structure or tight-glycogen binding to enhance glycogen synthesis in certain cellular conditions. In conclusion, they catalyse several rounds of elongation and they all consist of a mechanism to retain the growing polysaccharide chain. The observation that similar structural features occur between processive GTs across the different kingdoms of life highlights the importance of further exploiting this knowledge (Table 1). Understanding the mechanism by which certain processive enzymes retain the growing glycan chain could be a possible key to interfering in important physiological processes, e.g. change texture of certain rice varieties (Li et al., 2016) or strategy for the conversion to healthy glycogen in Type-2 diabetes (Sullivan et al., 2011) (Table 1). Striking examples for this, are the site-directed engineering strategies for hyaluronan synthase in an attempt to change and control chain-length.

2.2. Engineering of hyaluronan synthase for increased product chain-length

An additional example to this list, are the class I processive hyaluronan synthases (HAS). These membrane-embedded β-glycosyltransferases (GT2 family) synthesise hyaluronic acid (HA) which is

composed of repeating disaccharide units of D-glucuronic acid (GlcA) and GlcNAc: $[GlcNAc(\beta1,4)GlcA(\beta1,3)]_n$ (Weigel et al., 2014; Yang et al., 2017). It is an essential component of the extracellular matrix and is important for cell signalling and wound repair (Nishida et al., 1999; Parker et al., 2009). For this reason, industrial HA production, mostly by bacterial fermentation, is an important and fast-growing market due to the different routes of applications in pharma and cosmetics (recently reviewed by (Gunasekaran et al., 2020)).

More specifically, class I of HAS elongates the growing glucan chain at the reducing end rather than the non-reducing end, which is the case for most glycosyltransferases (Prehm, 2006; Tlapak-Simmons et al., 1999; Weigel et al., 2014). This reducing end polymer elongation occurs by an inverting mechanism where the polymer-UDP acts as a donor substrate for nucleophilic attack of GlcNAc-UDP or GlcA-UDP as acceptor to create the glycosidic linkage (Agarwal et al., 2019; Maloney et al., 2022; Weigel, 2015; Weigel et al., 2017). In contrast to cellulose, chitin, starch or glycogen polymers, the importance of HA chain size on structural functions and cell signalling processes is better known (D'Agostino et al., 2017; Jiang et al., 2005; Sahoo and Schwille, 2013; Stern et al., 2006). HA interacts with several membrane receptors, such as CD44, RHAMM and Toll-like receptors (TLR) (Misra et al., 2015). High-molecular-mass HA promotes survival and repair during epithelial cell injury and has a general anti-inflammatory and anti-angiogenic effect (Jiang et al., 2005). Low-molecular-weight HA is associated with an increase in total blood vessel volume after bone marrow ablation (Raines et al., 2011). In general, the lower the HA size, the higher the pro-inflammatory effect (D'Agostino et al., 2017). Understanding these processes creates opportunities to intervene in several pathologies such as chronic inflammation or cancer. HA chain size is dependent on several factors: substrate concentration, hydrolysis by hyaluronidases but also the HAS structure (Agarwal et al., 2019; Maloney et al., 2022; Yang et al., 2017).

Similar to the discussed processive β-glycosyltransferases CeS and Chs, HAS forms a transport channel to translocate HA and contains the same conserved regions (Table 2) situated in so-called sub-structural elements (SSE) (Agarwal et al., 2019). More recently, (Maloney et al., 2022) determined cryo-electron microscopy structures of HAS from chlorella virus (CvHAS) in several primed and bound states. This unique work provides mechanistic insights into the human HAS2, as CvHAS and humans HAS2 share approximately 45% sequence similarity. Similar to Chs, CvHAS generates in situ the GlcNAc primer by substrate hydrolysis. What is of particular interest here is that GlcNAc priming is essential to create a continuous TM channel (Fig 5a). In this way, an exit path for the growing HA-chain is formed, similar to Ces (Maloney et al., 2022). In analogy to the gating loop of Ces, CvHAS consists of a priming loop repositioning the substrate towards the acceptor sugar. In conclusion, (Maloney et al., 2022) have shown that Cv-HAS, and by extension other Class I HAS, work according to a similar step-by-step translocation mechanism as in Ces.

Next to this work, several mutant Class I HAS are reported for which specific residues have an influence on molecular weight of the HA polymer (Agarwal et al., 2019; Kumari et al., 2006; Yang et al., 2017; L. Zhang et al., 2016). Next to the conserved QxxRW motif, HAS consist of two conserved polar amino acids that, upon mutation, have an impact on HA-product size (Kumari et al., 2006). (Yang et al., 2017) discovered that the last nine C-terminal residues in the HAS of *Streptococcus equisimilis* are crucial for catalytic activity although they are not situated in the GT catalytic site and this by the formation of bidentate hydrogen bonds between arginine and the HA chain. They even successfully succeeded in enlarging the HA product size. Changing several crucial leucines into a lysine or arginine probably

enhanced the interaction between the C-terminus and the HA chain (Fig. 5b). However, this increased interaction affected HA synthesis efficiency in a negative way suggesting that a delay in HA translocation increases HA product size but decreases total HA titres (Yang et al., 2017). In addition, an increased HA chain length was obtained by the substitution of several polar residues, at the N-terminus, with hydrophobic amino acids. In this study they postulated that an increased hydrophobicity of the total HAS would be beneficial for the strength of the catalyst-polymer interaction (Mandawe et al., 2018).

The example of HAS complements the results of other processive β-glycosyltransferases in that the interaction between several non-catalytic sites and the growing glucan chain is an important key in processivity but can be a crucial position in order to change molecular weight and hence biological function. Although HAS is an interesting example of how processivity can be controlled, more opportunities lie in the GT2 family of enzymes which show striking similar structural features and hints to control product molecular weight. As shown below, multiple reports point towards strongly conserved regions and domains in the GT2 family which are implicated with processive mechanisms.

2.3. Conserved GT2 domains

Bioinformatic analysis, using structural and sequence alignments, revealed a consensus topology for the GT-A fold, shared by β-polysaccharide synthases (GT2 family). Interestingly, GT-A adopting enzymes share a highly variable region for which neither a consensus structure nor a conserved amino acids pattern can be assigned to (Romero-García et al., 2013). Next to this topology, GT2 enzymes consist of six short conserved regions important for glycosyl transfer (Table 2).

Table 2. Conserved regions in β-polysaccharide synthases. *Specific region for hyaluronic acid synthases.

Region	Consensus sequence	Function
1	DDGS	UDP-binding
2	GKR*	
3	D(S/A)DT	Donor saccharide-binding
4	Gxxxx(Y/F)R	
5	(G/S/T)(E/D)DRxx(T/S)	Acceptor saccharide-binding
6	Q(Q/R)xRW	Product-binding

These conserved regions are located in the cytoplasmic region between two membrane-spanning regions (Dorfmueller et al., 2014; Gohlke et al., 2017; Kamst et al., 1999; Kamst and Breek, 2000; Kamst and Herman, 1999; Morgan et al., 2014; Sobhanifar et al., 2016; Yang et al., 2017). To confirm the GT-A consensus structure and conserved domains in the GT2 family, a multiple sequence alignment (MSA) of several GT2 enzymes - only GT-A domain - with solved 3D structure was performed. Both prokaryotic and eukaryotic GT2 enzymes were added to the alignment (Fig. 6). An important conserved motif in GT-A fold enzymes is the DxD motif (region 3 in Table 2) and primarily interacts with the phosphate groups of the nucleotide donor saccharide using a divalent cation. This motif is always found at the same location in the structure: in a short loop between two small β-strands. In Fig. 6 the DxD motif is situated between β_4 and $\beta_{4'}$. This motif is found in both processive and non-processive enzymes (Dorfmueller et al., 2014; Götting et al., 2004; Morgan et al., 2014; Sobhanifar et al., 2016; Wiggins and Munro, 1998). However, UDP-binding is not only mediated by the DxD motif but other residues play a role in nucleotide binding (region 1 and 2 in Table 2) (Charnock et al., 2001; Götting et al., 2004; Li et al., 2001). Region 5 probably facilitates acceptor deprotonation. Interestingly,

the presence of a glycine or threonine/serine in this region could indicate specificity for, respectively, GlcNAc or Glc (e.g. 'TED' in CeS or 'GDD' in hyaluronan synthase) (Kamst and Herman, 1999; Morgan et al., 2014, 2013; Oehme et al., 2019).

Of particular interest is the well-conserved QxxRW sequence (region 6) in several processive and membrane-embedded β-glycosyltransferases. Up till now, it is believed that the QxxRW motif acts as product-binding site, retaining it for further addition of monomers, such as GlcNAc or Glc (Dorfmueller et al., 2014; Kaur et al., 2016; Morgan et al., 2014, 2013; Oehme et al., 2019; Pérez-Mendoza et al., 2017). Of specific importance is Trp (W in the QxxRW motif), enabling a strong carbohydrate-π stacking interaction and stabilising translocation. The importance of the QxxRW motif in stabilising the sugar ring, was again proven by (Maloney et al., 2022), and in this way guiding the growing HA-chain further into the continuous TM channel.

Next, not only the QxxRW motif but also the size and shape of the transport channel in enzymes such as CeS, Chs or HAS are important to prevent premature release of the glucan chain (Maloney et al., 2022; McNamara et al., 2015; Oehme et al., 2019). The size of the channel is decreased due to the presence of aromatic residues, enabling important C-H-π interactions and hydrogen bonds interactions (Knott et al., 2016; Maloney et al., 2022; Morgan et al., 2014; Oehme et al., 2019). Interestingly, it seems that next to maintaining the product in the channel, the bulkiness and amount of this conserved aromatic residues in the TM channel influence the functional specificity of certain β-glucan synthases: (1,3)-β-glucan or (1,4)-β-glucan specificity for respectively curdlan synthase and cellulose synthase. At the end of the TM channel, where the glucan enters the extracellular environment, residues are less conserved and ensure that the TM channel is long enough to cross the membrane (Oehme et al., 2019). Additional site-directed mutagenesis studies even revealed an extra important conserved motif in CeS, FFCGS (Sun et al., 2016). This study proposed that the cysteine side chain in this motif associates with surrounding aromatic side chains by a sulphur-arene interaction. In this way, the carbonyl group of the cysteine is correctly positioned to interact with the molecular terminus of cellulose and mediates successive rounds of glycosyl transfer (Sun et al., 2016). In CvHAS, the HA-chain also interacts with predominantly conserved polar and charged side chains. Interestingly, the GlcNAc groups in HA are mainly coordinated by polar and aromatic residues. In contrast, positively charged arginine amino acids coordinate GlcA groups. In this way, the enzyme dynamically coordinates the HA-chain inside the TM channel and ensures an alternating GlcNAc-GlcA distribution (Maloney et al., 2022).

Knowledge of these GT2-conserved and less conserved residues, e.g. carbohydrate binding domains, charged grooves or specific residues in the TM channel, can contribute to interesting protein engineering attempts to change product specificity (Table 1).

Table 1.

Overview of the discussed GTs with (partly) solved processivity mechanism. Possibilities for engineering strategies or regions important as antifungal targets are described. GT = glycosyltransferase, TM = transmembrane

Enzyme	GT family	Product	Function/Importance	Mechanism of processivity	Possibilities to intervene
Cellulose synthase (BcsA)	GT2	(Bacterial) Cellulose (1,4-β-linkage)	**Industry:** veterinary foods, paper, cosmetic and pharma (Lavanya et al., 2015; Swingler et al., 2021) **Fundamental**: Learn more about homologous enzymes	- TM channel with C-H-π-interactions (Oehme et al., 2019) - QxxRW motif (Morgan et al., 2014) - FFCGS region (Sun et al., 2016)	Site-directed mutagenesis towards aromatic residues in TM channel to change functional specificity
Chitin synthase (Chs2)	GT2	Chitin (1,4-β-linkage)	**Industry:** fibers, hair care or waste water treatment (Pokhrel et al., 2016) **Fundamental:** Development of antifungal drugs	- TM channel (Dorfmueller et al., 2014; Orlean and Funai, 2019) - Chitin bursts upon donor depletion (Orlean and Funai, 2019)	- Site-directed mutagenesis of TM channel to change product specificity - Targets that block the TM channel
Bacterial glucan synthase	GT2	Curdlan (1,3-β-linkage)	**Industry**: gelling agent or food additive (McIntosh et al., 2005) **Fundamental**: Improved knowledge of glucan synthases in other kingdoms	- TM channel with C-H-π-interactions (Oehme et al., 2019) - QxxRW motif (Oehme et al., 2019)	Change TM channel size: curdlan synthesis with altered chain length
Fungal glucan synthase	GT48	Glucan (1,3-β-linkage)	**Industry**: Functional food ingredient, immunity booster (Zhu et al., 2016) **Fundamental**: Development of antifungal drugs	Not known but probably similar to bacterial glucan synthase (Chhetri et al., 2020)	Switch focus of potential inhibitors of accessory enzymes to glucan synthase
WTA β-glycosyltransferase TarS	GT2	β-O-GlcNAcylated WTA (1,4-β-linkage)	**Industry**: / **Fundamental**: Development of anti-methicillin resistant drug design (Sobhanifar et al., 2016)	Trimerisation domain with tandem carbohydrate binding domains (CBM) (Sobhanifar et al., 2016)	Inhibitors towards CBM domains: block chain elongation
WTA α-glycosyltransferase TarM	GT4	α-O-GlcNAcylated WTA (1,4-β-linkage)	**Industry**: / **Fundamental**: Development of anti-methicillin resistant drug design (Sobhanifar et al., 2015)	Positively charged groove that binds the negatively charged product (Sobhanifar et al., 2015)	Inhibitors with high affinity towards the binding groove
Starch synthase	GT5	Amylose or Amylopectin (1,4-α-linkage)	**Industry**: Calorie source for many animals + food and paper-making (Jobling, 2004) **Fundamental**: Model for homologous enzymes + Understanding nutrition-related diseases (Gilbert, 2011)	Extra loops surrounding the reducing-end glucose (Baskaran et al., 2011; Nielsen et al., 2018)	Alter starch composition by targeting accessory loops through site-directed mutagenesis, e.g. change texture (Li et al., 2016)
Glycogen synthase (pro- and eukaryotic)	GT5	Glycogen (1,4-α-linkage)	**Industry**: food and paper-making **Fundamental**: Understanding of nutrition-related diseases (obesity or diabetes)	Multiple non-catalytic high affinity glycogen-binding sites (Baskaran et al., 2011; Díaz et al., 2011; Sheng et al., 2009)	Increase/Decrease tight glycogen association by site-directed mutagenesis in these glycogen-binding sites: conversion to healthy glycogen (Sullivan et al., 2011)

3. Controlled processivity

3.1. Counting mechanism in pathogen-related enzymes

PglH is defined as a semi-processive and retaining glycosyltransferase belonging to the GT4 family (Ramírez et al., 2018; Troutman and Imperiali, 2009). PglH is an essential enzyme in the protein *N*-glycosylation process of the Gram-negative pathogen *Campylobacter jejuni*. This protein glycosylation aids in virulence towards the human gut mucosa, causing gastroenteritis and food-borne illness (Ketley, 1997; Torres et al., 1998; Warner et al., 2002). Studies with a mutated *pgl*H gene severely affected adhesion and invasion of human epithelial Caco-2 cells (Karlyshev et al., 2004). Protein *N*-glycosylation requires a lipid-linked oligosaccharide (LLO) for transfer of the glycan to the protein. This process uses several glycosyltranferases: PglC, A, J, H and I (Glover et al., 2006). PglC mediates the first step and catalyses the linkage of uridine 5'-diphosphobacillosamine (UDP-Bac) to undecaprenyl phosphate (Und-P) to create the intermediate undecaprenylpyrophosphate-linked Bac (Und-PP-Bac). Next, PglA, J and H catalyse the transfer of five terminal *N*-acetylgalactosamine (GalNAc) units. Interestingly, PglA and PglJ transfer each a single GalNAc in contrast to PglH which catalyses three α-1,4 GalNAc units (Fig. 7a). PglH differs from the other discussed processive enzymes (GT2, inverting, and GT5, retaining) in that it is classified in the GT4 family which follows a S_Ni-like mechanism producing a retained glycosidic linkage. (Ramírez et al., 2018) investigated the counting mechanism behind this semi-processive GT. They discovered that PglH contains an amphipathic helix with three positively charged side chains that bind the pyrophosphate group of the LLO substrate and thus limits the addition of GalNAc units to three as a longer LLO substrate cannot fit into the active site (Fig. 7b) (Ramírez et al., 2018; Troutman and Imperiali, 2009).

Two other pathogen-related GTs, Galactofuranosyltransferase 1 and 2 (GlfT1 and 2), are described in literature as being processive. GlfT1 and 2 are implicated in the biosynthesis of the mycobacterial cell wall and full understanding of the synthesis mechanisms underlying cell wall formation in mycobacteria could broaden the array of targets and new drugs in tuberculosis treatment. Microbial arabinogalactan is an essential structural heteropolymer in the mycobacterial cell wall (Alderwick et al., 2008; Belánová et al., 2008; Mikušová et al., 2006). The galactan part is a homopolymer of around 20 to 40 galactofuranose (Galf) residues which are linked by alternating β-(1,5) and β-(1,6) linkages. These Galf residues are attached through an α-L-rhamnopyranosyl-(1,3)-α-D-*N*-acetylglucosamine-1-phosphate disaccharide (linker saccharide) to peptidoglycan. Finally, several Galf residues are added with mycolated arabinan domains. Altogether, the structure is called mycolyl-arabinogalactan-peptidoglycan (mAGP). GlfT1 and GlfT2 are two important GTs which transfer Galf residues to the linker saccharide using UDP-Galf as donor (Janoš et al., 2020; May et al., 2009; Poulin and Lowary, 2016; Rose et al., 2006). Both enzymes are classified in the GT2 family with inverting reaction mechanism. Literature describes both enzymes as being bifunctional: they can both catalyse the formation of β-(1,5) and β-(1,6) glycosydic linkages. GlfT1 initiates galactan synthesis on the linker saccharide by the transfer of two Galf residues from the UDP-Galf donor (Belánová et al., 2008). GlfT1 is proven to be a bifunctional enzyme, however until today, the question if this happens in a distributive or processive manner, is not yet answered. In contrast, (May et al., 2009) proved the ability of GlfT2 to add multiple Galf residues in a full processive manner: without releasing the intermediate product. Additionally, they postulated the presence of a lipid-binding secondary site using the substrate lipid substituent as a tether: increasing lipid length resulted in products with higher-molecular weight (Levengood et al., 2011; May et al., 2009). However, a few years later, the solved GlfT2 crystal structure did not reveal a lipid-biding site. Therefore, a new hypothesis was postulated by (Poulin and Lowary, 2016): a central cavity, formed by the GlfT2 homotetramer, is filled during chain elongation. Similar to PglH or NodC (chitin oligosaccharide synthase, see below), further polymerisation is prevented due to steric hindrance. In addition, the relative positioning of the

substrate lipid substituent influences the GlfT2 catalytic turnover (Yamatsugu et al., 2016). Structural and sequential knowledge of the GlfT2 polymerisation mechanism can result in the proposition of specific GlfT2 inhibitors for the production of deviated galactan and thus detrimental defects in the cell envelope of mycobacteria. In more detail, (Justen et al., 2020) already generated a GlfT2 variant that produced a reduced galactan chain length which directly affected cellular physiology and infectivity of *Mycobacteria*.

Two other bifunctional enzymes that could be categorised as processive are Alg2 and Alg11. Both are mannosyltransferases (GT4) implicated in the dolichol pathway which are highly conserved in all eukaryotes (Absmanner et al., 2010; Kämpf et al., 2009; O'Reilly et al., 2006). Dolichol is an important factor in *N*-glycosylation of proteins as it functions as a membrane anchor for the formation of a tetradecasaccharide (Glc_3Man_9-$GlcNAc_2$) which is transferred to certain asparagine residues (*N*-glycosylation) on nascent polypeptide chains and this at the endoplasmic reticulum (ER). *N*-linked glycans play very important roles going from cell signalling, protein stability, solubility and folding, cell structure and transport, protein degradation, etc. (Helenius and Aebi, 2004, 2001). First, Alg2 performs an α-1,3-mannosylation followed by an α-1,6 mannosylation using $Man_1GlcNAc_2$-PP-Dol as substrate and UDP-mannose as donor. Alg11 is responsible for the next two α-1,2-mannosylations yielding Man_5-$GlcNAc_2$-PP-Dol (Kämpf et al., 2009). Completion towards the tetradecasaccharide is carried out by several consecutive mannosyl and glucosyltransferases (Frank and Aebi, 2005). However, how the dual activity is regulated and if Alg2, Alg11 or GlfT1 have a way to hold on to the growing glycan chain is still unknown. A possible hint for further investigation could be found in the mechanism of a bifunctional glycosyltransferase, dual glycosyltransferase 1 (dGT1), which transfers Glc and GlcNAc residues to Fap1, a well-studied serine-rich repeat glycoprotein (SRRP). SRRP is required for bacterial adhesion and biofilm formation in *Streptococcus parasanguinis* (H. Zhang et al., 2016). dGT1 is a hybrid protein consisting of two functional domains: the N-terminal DUF1792, a novel GT-D type glycosyltransferase, and C-terminal dGT1 (CgT), a classic GT-A type glycosyltransferase (Zhang et al., 2014). However, this two-domain organisation seems to only exist in streptococcal species and likely does not explain the mechanism of bifunctional and one-domain proteins such as Alg2.

3.2. Counting mechanism in the rhizobial enzyme NodC

In contrast to the polymer-producing processive GTs, NodC is described to be a semi-processive chitin oligosaccharide synthase that, in nature, is part of the *nod*ABC cluster in rhizobial species. This *nod* gene system codes for the biosynthesis of chitin-like signal Nod factors. The latter are lipo-chitin oligosaccharides (LCOs) which control several key processes in symbiosis between *Rhizobia* species and *Leguminosae* plants (Chirak et al., 2016; Limpens et al., 2015; Spaink et al., 1993). NodC is an *N*-acetylglucosaminyltransferase involved in the synthesis of the LCO backbone, whereas NodA and NodB are involved in the acylation and de-*N*-acetylation of the non-reducing terminal *N*-acetylglucosamine, respectively (Poinsot et al., 2016). The transcription factor NodD, activated by secreted plant flavonoids, recognises the so-called *nod* boxes which are regulatory elements of the *nod* genes (Peck et al., 2006). Interestingly, a wide array of LCOs exist: depending on the LCO decorations (acylation, arabinosylation, fucosylation, etc.) and backbone length specific recognitions between organism and host plant occurs (Berg et al., 2016; Phour et al., 2020). The LCO backbone, synthesised by NodC, is mostly consisted of a chitintetraose or -pentaose backbone (Barny et al., 1996; Zhang et al., 2007). Similar to other GT2 family enzymes, chitin oligosaccharide (COS) synthesis occurs by the sequential addition of monosaccharides (GlcNAc) to the growing oligosaccharide chain. NodC does not use free COS molecules as acceptors if they are available in the reaction mixture. Acceptor GlcNAc and donor UDP-GlcNAc are sufficient to initiate synthesis (Kamst et al., 1999). A detailed

description about COS synthesis by NodC is given by (Dorfmueller et al., 2014): to establish a β-glycosidic linkage, GlcNAc is first activated by the catalytic base Asp-241 to perform a nucleophilic attack on the anomeric carbon of UDP-GlcNAc (Bi et al., 2015).

In summary, NodC has two important domains: one which binds the UDP-sugar and the other which binds the acceptor (Charnock et al., 2001; Dorfmueller et al., 2014; Saxena et al., 1995). Next, NodC is fixed into the cell membrane (John et al., 1985; Schmidt et al., 1988, 1986). For a long time, research assigned NodC to the outer, inner and even both membranes (Barny and Downie, 1993; Hubac et al., 1992; Schmidt et al., 1986). In recent years, NodC is proven to be present in the inner-membrane, allowing the catalytic domain to face the cytoplasm (Dorfmueller et al., 2014). The NodC amino acid sequence shows three highly hydrophobic regions - one located at the Nt and two at the Ct - with two intermediate domains. The Ct region plays the dominant role in correct membrane topology. Missing (part of) this Ct results in incorrect membrane insertion and hence loss of function (Barny et al., 1996; Schmidt et al., 1986). Therefore, it is believed that the same localisation and topology occurs for heterologous NodC expression in a host such as *E. coli*. Next to elucidating the catalytic mechanism, (Dorfmueller et al., 2014) did prove the inner-membrane localisation of NodC using a combination of a green fluorescent protein (GFP) and PhoA C-terminal fusion proteins. Between the two central hydrophobic regions, a large hydrophilic region is present to efficiently interact with the cytoplasmic NodA and NodB enzymes in *Rhizobia* (Barny et al., 1996; Dorfmueller et al., 2014).

In literature, there exist some indications for NodC specific regions that are responsible for this difference in product chain length. The Ct region of maximally 164 amino acids would be the main factor for the tetraose backbone of *S. meliloti*. If the C-terminal region of *Mesorhizobium loti* (*M. loti*) - producing mainly chitinpentaose - is exchanged for the C-terminal region of *S. meliloti*, *M. loti* starts producing chitintetraose in excess. However, the reverse was not observed: it was not possible to convert the chitintetraose producing strain to a chitinpentaose one (Kamst and Breek, 2000). A possible explanation is the β-strand around residue 300 in the C-terminal region of NodC. The shorter this β-strand, the shorter the chain length (Kamst and Breek, 2000). This β-strand is closely located to the highly conserved region Q(Q/R)XRW which is thought to bind the product chain and have an influence on chain length (Saxena and Brown, 1997). Until today no crystal structure is available for NodC enzymes, making it challenging to fully understand mechanisms behind producing chitin oligomers. In order to study NodC enzymes on a structural and mechanistic level, (Dorfmueller et al., 2014) created an homology model of NodC from *S. meliloti* SM11 based on the published BcsA cellulose synthase. They hypothesised that the predicted product-binding site is limited by two amino acids, Arg-349 and Leu-19, to five binding sites, whereas the cellulose synthase structure forms a transport channel through the membrane formed by the transmembrane domains (Ser-111 and Ser-459) (Fig. 8). This suggests that increasing space in the catalytic cleft towards the formation of a transport channel through the membrane could increase the backbone length. To this end, bulky side chain amino acids, sterically blocking further chain elongation, should be shortened (Dorfmueller et al., 2014).

3.3. Controlling chain-length using a protein acceptor

A common definition for processive GTs is the consecutive addition of UDP/ADP-activated glycosyl donors to a glycosyl acceptor without releasing the acceptor substrate. However, multiple GTs exist that do not use a glycosyl group but a protein as acceptor. The (semi-)processive character of several protein GTs allows the creation of densely glycosylated proteins which often play an important role in signaling and communication (Yakovlieva et al., 2021). In general, protein GTs can be divided into two categories based on the glycosylation position, i.e. *N* or *O*-linked glycosylation. A striking example of each category are glycogenin and adhesin glycosylation, respectively.

Glycogenin is classified in family 8 and adds approximately 6-10 α-1,4-linked glucose units to itself using UDP-Glc as a donor (Chaikuad et al., 2011; Smythe and Cohen, 1991). In this way, the complex of glucose residues attached to glycogenin serves as a primer for further bulk glycogen synthesis by other enzymes such as the discussed processive glycogen synthase and branching enzymes. Deficiencies in glycogen formation or storage is often caused by molecular changes in these enzymes that can result in metabolic diseases and cancer (Adeva-Andany et al., 2016; Zois et al., 2014). Glycogenin works as a dimer of two subunits which gives the enzymes the ability to form two longer nascent maltosaccharide chains in an intersubunit mode, although it has been proven that the enzyme is able to synthesise the glycogen primer without prior dimerisation (Issoglio et al., 2012). Catalysis is mediated by loop movement of an α-helix by which lid closure is important to fixate donor and acceptor. Recent work by (Bilyard et al., 2018), suggested a reaction mechanism of three phases that reflect the transition from an intra-monomeric to an inter-monomeric form (Fig. 9). From the moment that the oligosaccharide chain passes the active site of its own monomer, the dimeric enzyme complex switches to an intersubunit mode to further extend the growing maltosaccharide chain (Bilyard et al., 2018). For this, crucial hydrogen-bonding interactions were investigated deep in the active site (Chaikuad et al., 2011). Extension of the maltosaccharide chain ceases if the chain passes the second active site (Bilyard et al., 2018).

As discussed throughout this review, the process of protein glycosylation and decoration is characterised by the combination of different GTs of which several are implicated in a processive (GlfT2), distributive (GflT1, Alg2 and Alg11) or semi-processive mechanism (PglH). The obtained complex glycoproteins often play an important role in efficient adherence and invasion of different cell types. A specific example is the glycosylation of high-molecular weight adhesins on the extracellular surface of *Heamophilus influenzae* as this is a first essential step in the adherence and infection of human epithelial cells causing problems in the upper respiratory tract (Turk, 1984). (Yakovlieva et al., 2021) studied the mechanism of the implicated *N*-GTs that generally catalyse the transfer of a large number of Glc residue to an asparagine residue on the adhesin creating a β-glycosidic linkage. They showed that two selected N-GTs from *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae*, respectively, displayed semi-processive behavior. Molecular dynamics simulations revealed a promiscuous substrate binding groove for both *N*-GTs which allows simultaneous sliding and hyperglycosylation of the substrate adhesin (Yakovlieva et al., 2021). Additionally, they proposed some acceptor binding sites which would prevent the product from leaving the binding groove. Mutating one of these sites already lead to the production of a more homogeneously glycosylated adhesin (Song et al., 2017; Yakovlieva et al., 2021).

A plethora of different processive protein GTs exist that are involved in the hyperglycosylation of adhesins (e.g. GtfA/GtfB enzyme complex or SdgB/SdgA transferases), autotransporters (e.g. Aah and TibC transferases), pili or flagella. Although their mechanism of catalysis if often completely clarified, only few or no structural hints still exist concerning their mode of (semi-)processivity (Yakovlieva and Walvoort, 2019). Therefore, these enzymes are not discussed in this review but remain of interest to further elucidate and interfere with the hyperglycosylation mechanism in pathogenic bacteria. The discussed examples glycogenin and *N*-GTs indicate the importance and potential of studying chain-length control in protein GTs.

3.4. In vitro versus in vivo set-up

All the examples, described above, indicate the role of processive and semi-processive enzymes in several metabolic pathways and how complete knowledge of the reaction mechanism can aid in the development of multiple therapeutic drugs or relevant industrial applications. Next to the specific reaction mechanism of these enzymes, it is also important to consider how the experimental set-up

or analysis technique is being performed as this can influence the final product. For example, it is generally accepted that Chs produces high-molecular-weight chitin due to the formation of a TM channel (Orlean and Funai, 2019). However, in vitro analysis of Chs suggested the formation of chitin oligosaccharides, in addition to chitin, due to premature chain termination or aberrant initiation (Gyore et al., 2014). Therefore, the use of (semi-)processive enzymes in a biocatalytic approach could be preferred over in vivo expression and this for the production of interesting oligosaccharides, e.g. cellooligosaccharides which can be wildly applied for the production of biosurfactans, hydrogels or prebiotic ingredients (De France et al., 2017; Karnaouri et al., 2019; Voon et al., 2016).

The β-1,4-galactosyltransferase LgtB was identified as a possible biocatalyst for the production of soluble cellooligosaccharides (Bulmer et al., 2020). This enzyme from *Neisseria meningitides* is best known as one of the key enzymes in the in vivo production of human milk oligosaccharides (HMOs) (Recently reviewed by (Faijes et al., 2019)). In this strategy, LgtB catalyses the conversion of the trisaccharide GlcNAc-β-1,3-Gal-β-1,4-Glc to lacto-*N*-neotetraose (Galβ-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc) (Dumon et al., 2006; Priem et al., 2002). In addition, co-expression of *nod*C (described below) with *lgt*B lead to the in vivo production of *N*-acetyllactosamine-containing oligosaccharides, which can act as a precursor for many complex carbohydrates (Bettler et al., 1999). Although LgtB has been described as a non-processive glycosyltransferase in vivo (GT25 family), (Bulmer et al., 2020) applied the enzyme for in vitro glucose polymerisation. Next to the polymerase activity, LgtB displayed activity towards multiple UDP-donor substrates such as UDP-Glc. In this way, a biocatalytic approach for glucose polymerisation was established with multiple reaction products as a result. Additionally, depending on length of incubation and UDP-Glc concentration different length and ratio of cellooligosaccharides were produced (Bulmer et al., 2020).

As described for HAS, site-directed HAS engineering resulted in the production of HA with increased product chain length. Although HA is often produced using recombinant microbial strains, this production route suffers from a broad product size distribution (Cheng et al., 2016). In contrast, enzymatic in vitro HA synthesis can be an alternative for production of size-specific and monodisperse HA (Eisele et al., 2018; Fu et al., 2017). To this end, (Eisele et al., 2018) developed a one-pot enzymatic synthesis of HA through nucleotide sugar regeneration. Additionally, chain length was controlled by variation of the acceptor substrate which was a HA oligosaccharide (Eisele et al., 2018).

Table 3.

Overview of the discussed GTs with the ability to control the final poly- or oligomer chain-length. Possibilities for engineering strategies or regions important as antifungal targets are described. GT = glycosyltransferase, TM = transmembrane

Enzyme	GT family	Product	Function/Importance	Mechanism of processivity	Possibilities to intervene
N-acetylgalactosamine transferase (PglH)	GT4	Penta-LLO (α-1,4-linkage)	**Industry**: / **Fundamental**: Blocking gastroenteritis-causing pathogens (Ketley, 1997; Torres et al., 1998; Warner et al., 2002)	Steric hindrance for additional GalNAc residues	Inhibitors that bind ruler helix
Galactofuranosyl transferase 2 (GlfT2)	GT2	Microbial arabinogalactan (β-1,5- and β-1,6-linkages)	**Industry**: / **Fundamental**: Development of new drugs in tuberculosis treatment (Alderwick et al., 2008; Belánová et al., 2008; Mikušová et al., 2006)	GlfT2 homotetramers forms central cavity causing sterical hindrance during chain elongation	Inhibitors that bind specifically to central cavity
α-glucosyl transferase Glycogenin	GT8	Glycogenin (α-1,4-linkage)	**Industry**: food and paper-making **Fundamental**: Understanding of nutrition-related diseases (obesity or diabetes) (Nawaz et al., 2021; Nitschke et al., 2017)	Dimeric enzyme complex forms path for the growing maltosaccharide chain	- Add bulky side chain amino acids close to the second active site - Inhibitors that block crucial hydrogen-bonding interactions
N-GT from - *Haemophilus influenzae* - *Actinobacillus pleuropneumoniae*	GT41	Hyperglycosylated adhesin	**Industry**: / **Fundamental**: Understand the mechanism of bacterial protein hyperglycosylation (Yakovlieva et al., 2021)	Sliding of the substrate along a promiscuous groove	- Decrease product-enzyme association strength (Song et al., 2017) - Identification of specific inhibitors towards sliding groove
N-acetylglucosaminyl transferase (NodC)	GT2	Chitin oligosaccharide (COS°	**Industry**: Microbial COS-production with applications in pharma, food, feed and cosmetic industry (Ling et al., 2018) **Fundamental**: Discovery of antifungal inhibitors (Dorfmueller et al., 2014)	Bulky side chain amino acids cause sterical hindrance	- Enlarge space in the catalytic cleft to increase backbone length - Specific inhibitors that bind and occupy catalytic cleft

4. Conclusions and perspectives

In recent years, the field of glycobiology has attracted plenty of attention as functional glycans, and more specifically specialty poly- and oligosaccharides, mediate a variety of recognition processes which are crucial to plant, animal and human health (Issoglio et al., 2012; Passi and Vigetti, 2019; Sobhanifar et al., 2016). In particular of interest are the enzymes, the GTs, involved in the biosynthesis of this group of molecules. Full insight into an enzymatic reaction mechanism can be a valuable asset in the discovery and development of antibiotic targets but can also create relevant leads in site-directed protein engineering strategies. In this review, we focused on a specific part of glycan-processing enzymes that synthesise high-molecular-weight polysaccharides: the processive GTs. More specifically, processivity is a way to produce long chain-length polymers, e.g. DP of > 6000 monomers (Chhetri et al., 2020), by binding tightly to the growing substrate. In general, these high binding affinities are accomplished in several ways: multimerisation of the enzyme to guide the growing glycan chain (Bilyard et al., 2018), formation of a TM channel to enclose the product (Maloney et al., 2022; Oehme et al., 2019) or large carbohydrate-binding domains to allow additional rounds of chain elongation (Sobhanifar et al., 2016). In these binding strategies, a remarkable difference has to be noted between membrane-embedded and cytoplasmic processive GTs. Integration of GTs in the membrane almost always means the formation of a TM channel by which crucial hydrogen bonds or C-H-π-interactions mediate functional specificity and prevent premature product release. In contrast, processive GTs not associated to the membrane are often involved in multimerisation with the formation of product binding grooves or non-catalytic carbohydrate binding domains which in a way can mimic the formation of a TM channel to prevent premature product release.

Next, the catalysis mechanism in these processive GTs is often mediated by the dynamic properties of the protein, e.g. presence of dynamic loops, transition from an open to closed state, etc. (Sobhanifar et al., 2015) postulated that this transition would create a motion that extends along the acceptor binding path which would aid to enzyme processivity. Therefore, research opportunities lie in further investigating the role of protein dynamics in processivity. On the other end of GT chain-length control continuum, stands the characteristic feature of distributivity. Although it was not thoroughly discussed in this review, exploitation of distributive GTs, in an in vitro context, can be of interest for the polymerisation of industrially relevant products, e.g. cellooligosaccharides (Bulmer et al., 2020). Of superior interest in this review, were the semi-processive enzymes which bear a reaction mechanism that lies in between strict processivity and distributivity.

Understanding the internal ruler mechanism in semi-processive enzymes can become an apparent protein engineering strategy and this for both industrial applications as for identification of therapeutic targets. Again, the study of protein dynamics in semi-processive enzymes could provide new insights into mechanistic features, e.g. performing long-scale molecular dynamics studies to identify flexible protein loops. Altering these dynamics by protein engineering could therefore lead to products with different chain lengths. Next, researchers must acknowledge the parallel chain-length controlling mechanisms over several GT families, e.g. counting mechanism in GT4 PglH compared to internal ruler mechanism in GT2 NodC, but also over the different kingdoms of life. The internal ruler mechanisms discussed in this review can serve as a first hint to perform site-directed mutagenesis at positions that are crucial for protein-enzyme interactions. Lead molecules in rational inhibitor drug design are often substrate-like and target the catalytic center of pathogenic-related GTs (Chen et al., 2017). However, as the poly- or oligosaccharide chain size can be critical for many physiological and pathophysiological conditions, an opportunity lies in the development of new targets that could obstruct or alter the number of chain elongation rounds and hence the enzyme's bioactivity.

Finally, studying processivity of GTs will significantly aid in elucidating the specific structure-function relationships of several long chain polysaccharides. It is already known that, for example, changing the molecular size of amylose or altering the molecular structure of glycogen results in different textural properties after cooking (Li et al., 2016) and can aid in the conversion from type-2 diabetes-related to healthy glycogen (Sullivan et al., 2011), respectively. The possibility to engineer or control the number of elongation rounds in a processive GT could significantly affect the product chain-length and therefore also the bioactivity of the resulting polysaccharide, an approach that was successfully exploited by (Yang et al., 2017). Consequently, engineering amylose or curdlan synthase could change the texture and hence bitterness of cooked rice or broaden the industrial applications of curdlan emulsifying agents, respectively. Additionally, engineering the full processive chitin synthase into a semi-processive one would enable the production of chitin oligosaccharides.

In this respect, in vivo heterologous expression of these GTs, relying on the endogenous activated sugar pool of the host cell, has recently garnered attention as a promising route for the biotechnological production of numerous oligo- and polysaccharides (recently reviewed by (Coussement et al., 2020)). Combined with the mechanistic details and resulting site-directed engineering strategies, a plethora of native and new-to-nature specialty carbohydrates will be developed that can offer a desired structure for a specific function**.**

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