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Processivity in Bacterial Glycosyltransferases

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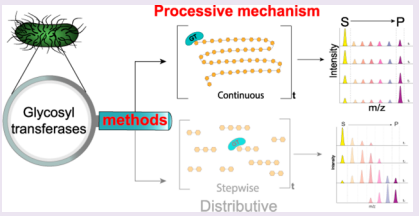
ABSTRACT: Extracellular polysaccharides and glycoproteins of pathogenic bacteria assist in adherence, autoaggregation, biofilm formation, and host immune system evasion. As a result, considerable research in the field of glycobiology is dedicated to study the composition and function of glycans associated with virulence, as well as the enzymes involved in their biosynthesis with the aim to identify novel antibiotic targets. Especially, insights into the enzyme mechanism, substrate binding, and transition-state structures are valuable as a starting point for rational inhibitor design. An intriguing aspect of enzymes that generate or process polysaccharides and glycoproteins is the level of processivity. The existence of enzymatic processivity reflects the need for regulation of the final glycan/glycoprotein length and structure, depending on the role they perform. In this Review, we describe the currently reported examples of various processive enzymes involved in polymerization and transfer of sugar moieties, predominantly in bacterial pathogens, with a focus on the biochemical methods, to showcase the importance of studying processivity for understanding the mechanism.



INTRODUCTION

High-molecular-weight polysaccharides are central constituents of the cell wall and extracellular matrix in all domains of life. Well-known examples include the homopolymers cellulose and chitin, and heteropolymers such as hyaluronic acid and other glycosaminoglycans. In eukaryotes, these extracellular polysaccharides are important for cellular integrity and cell−cell communication, while in bacteria they are part of the cell wall, membranes, capsule, and/or biofilm that forms as a defense mechanism from the host or a potentially hostile environment. Similar to the enzymes working on other natural biopolymers, such as DNA polymerase, protein translation by the ribosome, and protein kinases, many glycan-processing enzymes that synthesize and degrade these polysaccharides have a distinct mechanistic feature in common: they display (a degree of) processivity. A processive mechanism (Figure 1A) is characterized by a high binding affinity of the enzyme for the (growing) product chain, so that it stays associated during multiple rounds of catalysis yielding longer polymers or more uniform modifications. On the other end of the spectrum is the distributive mechanism (Figure 1B), where the enzyme performs one round of catalysis during every single binding event, which typically leads to a distribution of products. In a sense, processivity is Nature's way of ensuring the desired polymer length is produced with high fidelity and accuracy. It is important, for example, in DNA synthesis, where the associated polymerases are found to bind tightly to the growing substrate and are highly processive.[1,2] Because only a few classes of enzymes are strictly processive or distributive, these mechanisms are the two opposite ends of a continuum. Most polymerizing enzymes display a certain degree of processivity (so-called "apparent processivity"), which may be influenced by the nature of the substrate, or external factors such as substrate concentrations or membrane translocation machinery, and can be seen as the actual processivity of the enzyme under certain conditions. An alternative description is the theoretical "intrinsic processivity" (*vide infra*).[3] Originally developed for nucleic acid polymerases, it is defined as the distribution of probabilities that the enzyme will stay associated and catalyze another reaction, rather than dissociate from the intermediate product.

Processive mechanisms have been identified in a multitude of enzyme families, notably the DNA polymerases, exonucleases,[5] ribosomal protein translation machinery,[6] ubiquitin ligases,[7−9] kinases,[10,11] motor enzymes like kinesin,[12] glycosidases,[13] and glycosyltransferases (*vide infra*). Interestingly, these enzymes catalyze chemically different reactions, such as polymerization, degradation, and decoration, but all share certain mechanistic and structural characteristics to be called "processive". For instance, there are several structural features that promote processivity,[14] including the ability to form an inclusion complex with the growing substrate by enzyme domain multimerization, or having a flexible polypeptide patch that closes upon substrate binding.[15] Alternatively, high binding affinities are accomplished by large acceptor substrate-binding grooves that also promote sliding of the growing chain along the template, as is the case with chitin hydrolases.[16]

Processivity is a challenging mechanistic feature to experimentally investigate, and researchers have developed manifold methods to determine the degree of processivity of a plethora of enzymes.[3] In the field of glycan-processing enzymes, seminal reports exist on the processivity of various glycosyltransferases and hydrolases, which have been reviewed recently.[17,18] Also glycan-modifying enzymes, such as alginate and dermatan sulfate epimerases, show processive characteristics.[19,20] In this Review, we focus on the enzymes involved in bacterial polysaccharide and glycoprotein synthesis. Polysaccharides,

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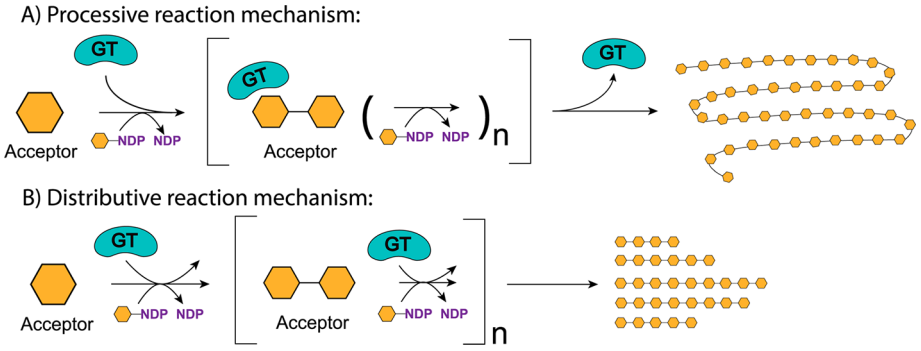

Figure 1. Schematic representation of the two ends of the catalytic mechanism spectrum. (A) In a processive reaction mechanism, the GT enzyme binds the acceptor substrate* and remains associated during multiple (n) rounds of catalysis; (B) In a distributive reaction mechanism, the GT enzyme releases the growing substrate after every reaction and rebinds to perform the next reaction (hexagon = carbohydrate, NDP = nucleotide-diphosphate, GT = glycosyltransferase, n = number of catalytic cycles). * In case the NDP moiety stays attached to the growing substrate (not drawn here), it can be involved in acceptor substrate binding during processivity, as is hypothesized for streptococcal hyaluronan synthase.[4]

glycans, and glycoconjugates are synthesized by dedicated glycosyltransferases (GTs), a family of enzymes that transfer a specific carbohydrate residue from a nucleotide- or lipid-pyrophosphate-activated donor to a certain carbohydrate, lipid, or protein acceptor substrate. This is an experimentally challenging class of enzymes that are all involved in the production of bacterial structures that are important in pathogenicity (Figure 2). Interestingly, bacteria-specific glycosylation of various crucial cell components presents a unique opportunity for species-targeted drug development because of their unique sugar structures and the resulting lack of cross-reactivity with human glycosylation systems.[21] As the bacterial glycans are assembled by bacteria-specific GTs, there is a wealth of possible targets for which inhibitors can be generated. However, because of the relatively young field of bacterial glycosylation, the precise molecular mechanisms of many bacterial GTs remain to be elucidated in detail to enable the rational development of inhibitors.[22]

To aid in the identification of processive characteristics in bacterial GTs, we discuss experimental methods to determine GT processivity and present current examples where these methods are used to study processivity of bacterial polymerizing GTs, semiprocessive GTs, and protein GTs. Also examples in which knowledge of processive behavior inspired inhibitor design are presented. As the characteristics of processive GTs are as diverse as the products they synthesize, it is hard to generalize and predict processivity. However, the integrative table at the end of this Review may assist in discerning processive features, which may be further identified using the methods described here.

METHODS TO STUDY PROCESSIVITY

The majority of the experiments that are aimed at unraveling processivity in enzymatic catalysis are performed *in vitro*. A mixture of the GT enzyme under study, together with the nucleotide-activated carbohydrate donor and a suitable acceptor (glycan, protein, lipid) are mixed. As the product distribution and kinetic profile will be significantly different between a processive and distributive mechanism, they are generally assessed at specific time points (Figure 3A).

Product Distribution. The degree of processivity is often deduced from the distribution of product lengths. For template-mediated polymerization, such as nucleic acid polymerization, the product length is determined by the template strand. In nontemplated polymerization, as is the case for glycosyltransfer reactions, the degree of processivity is largely determined by the binding affinity of the enzyme to the growing chain or resulting product. Analysis of the glyco- or proteoforms present at certain time points can provide insight into processivity/distributivity (Figure 3A). In the case of a processive enzyme, a gradual decrease of acceptor substrate and concomitant fast increase of product length may be observed over time, while the levels of intermediate products remain marginal (Figure 3B). A distributive process will yield a distinctly different pattern, with a rapid disappearance of the acceptor substrate and simultaneous appearance of several "early" glyco- or proteoforms that are all gradually converted over time. Processivity is also inferred by prolonged persistence of unmodified acceptor substrate, which may reflect the high affinity of the enzyme for its substrate and/or occurrence of enzyme−substrate covalent intermediate. Visualization of the product distribution is most commonly performed using techniques such as gel electrophoresis (SDS or native PAGE), thin-layer chromatography (TLC), and (liquid-chromatography-coupled) mass spectrometry (LC-MS).

Kinetics. A useful method of determining the degree of processivity in a quantitative manner is by measuring the kinetic parameters k_{off} and k_{cat}. One of the hallmarks of processivity is a strong enzyme−substrate (enzyme-intermediate) association, which can be converted into numerical values of the dissociation rate (k_{off}), whereas k_{cat} indicates the efficiency of acceptor substrate turnover. Combined in a formula (Figure 3C), these parameters give the intrinsic processivity P^{intr} of the enzyme as the average number of catalytic acts performed before dissociation.[3] k_{cat} is generally measured by performing enzyme kinetics analysis, and k_{off} can be estimated using techniques such as isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), and biolayer interferometry. Another potential kinetic feature of processive enzymes is an apparent **lag phase** at early reaction times. This lag is attributed to the slow formation of the short products, so-called "primers" at the beginning of the reaction, for which the enzyme has still a low affinity. When the enzyme has a higher affinity for the partially modified acceptor substrates as compared to the nonmodified substrate, subsequent catalysis increases the substrate affinity, which induces the processive fast phase and increases enzymatic turnover rate and product formation.

Single Hit. Varying the enzyme-to-substrate ratios may lead to further proof of processive behavior. Under so-called "single-hit conditions", the acceptor substrate is used in large excess compared to the enzyme (typically in 1:100 or 1:500 ratio). A distributive enzyme will quickly generate "early" products, since there is a higher chance of binding an unmodified substrate molecule upon dissociation rather than an intermediate molecule (Figure 3A, right). In contrast, processive enzymes will remain associated with the bound acceptor substrate and modify it to completion (Figure 3A, left). Therefore, the presence of a final product under single-hit conditions is indicative of processivity.

Distraction Assay. Another method that is linked to enzyme−substrate association is the distraction assay (Figure 3D). As is evident from the name, the enzyme is "distracted" from the reaction it is performing by the addition of a new batch of (labeled) acceptor

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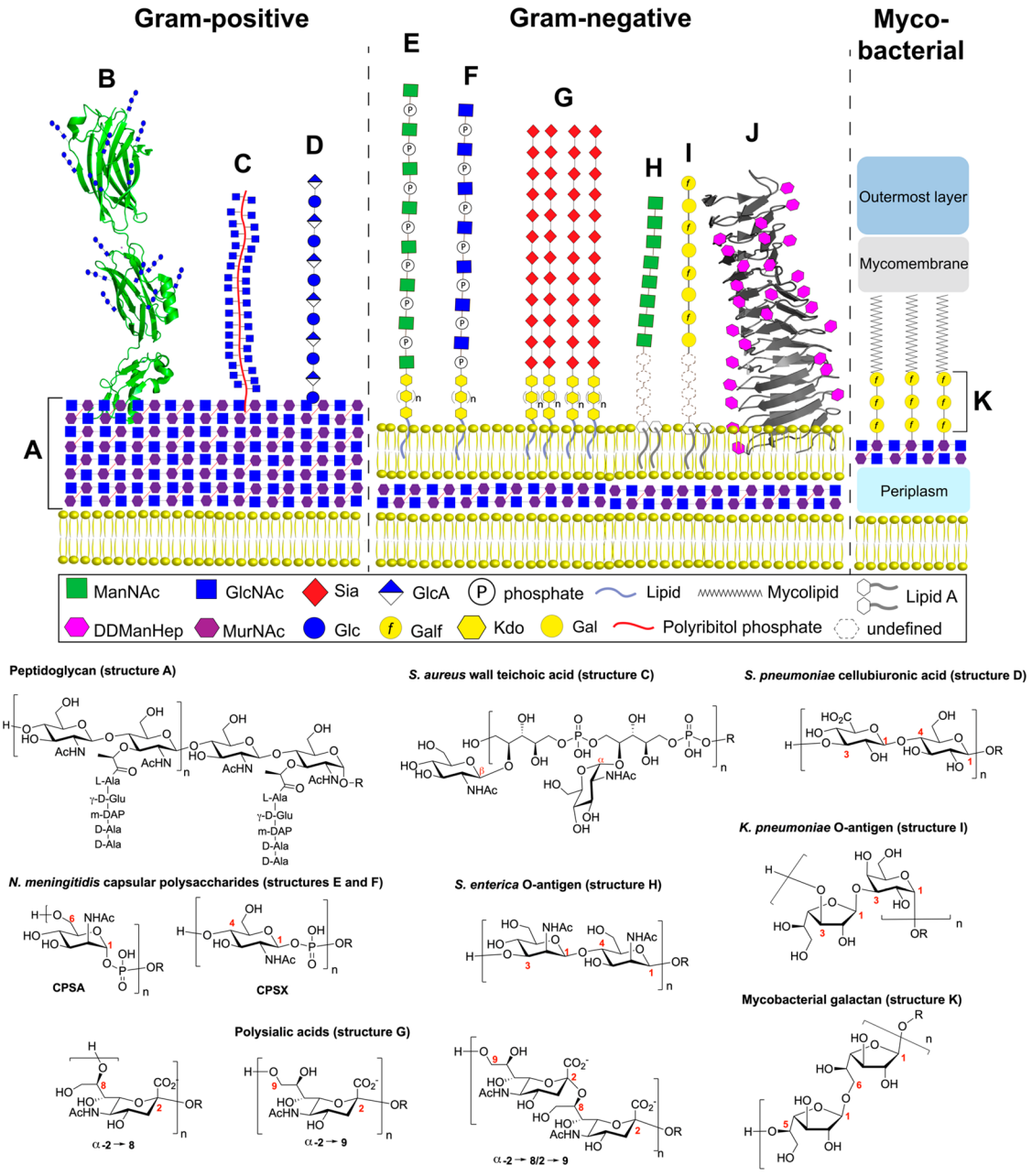

Figure 2. Schematic picture of bacterial cell walls showing the polysaccharides and glycoproteins discussed in this Review, with their chemical structures underneath. (A) peptidoglycan, (B) GspB adhesin, (C) wall teichoic acids, (D) cellubiuronic acid CPS, (E) CPSA, (F) CPSX, (G) polysialic acid, (H) and (I) O-antigens, (J) TibA autotransporter, (K) galactan.

substrate or inhibitor (also called a "pulse-chase" experiment). The product distribution analysis of a processive enzyme will reveal that when the enzyme is still working on the first batch of substrate, addition of a new batch will not lead to enzyme distraction. As a result, the majority of final products will not contain the label (Figure 3D, left). In the case of a distributive enzyme, the opposite effect is observed. Because the enzyme does not stay associated with the growing substrate or intermediates in-between catalysis rounds, both first and second batches of substrate will have equal access to the enzyme, creating products both with and without the label (Figure 3D, right).

As apparent from the examples presented below, the conclusion that a certain enzyme is processive is often challenging to draw, and involves performing several experiments using complementary methods. In general, the first hint of a processive mechanism is the fact that a single GT is involved in polymerization or multiple modifications of its substrate. Processivity is often inferred from enzyme similarity to other processive GTs in sequence, fold, function, or its association with the translocation machinery. Subsequently, researchers generally start by studying the structural features of the active site and substrate binding motifs, or by determining the product profile from the *in vitro* reaction. These experiments can be further supported quantitatively by measuring the dissociation constants and kinetic parameters to prove tight enzyme−substrate binding and calculate intrinsic processivity values, respectively. When the results of the performed experiments are ambiguous or paint a more complex picture (e.g., with semiprocessive enzymes), more advanced methods can be used to prove the mechanism. For instance, if an inhibitor or alternative substrate is known for the enzyme of interest, it can be used in a distraction assay (pulse-chase experiment) to prove the processive behavior. Alternatively, labeled substrate can be used in the same way to (dis)prove the mechanism. Generally, for most GTs multiple methods are used to prove processivity and unravel the inherent reasons for it.

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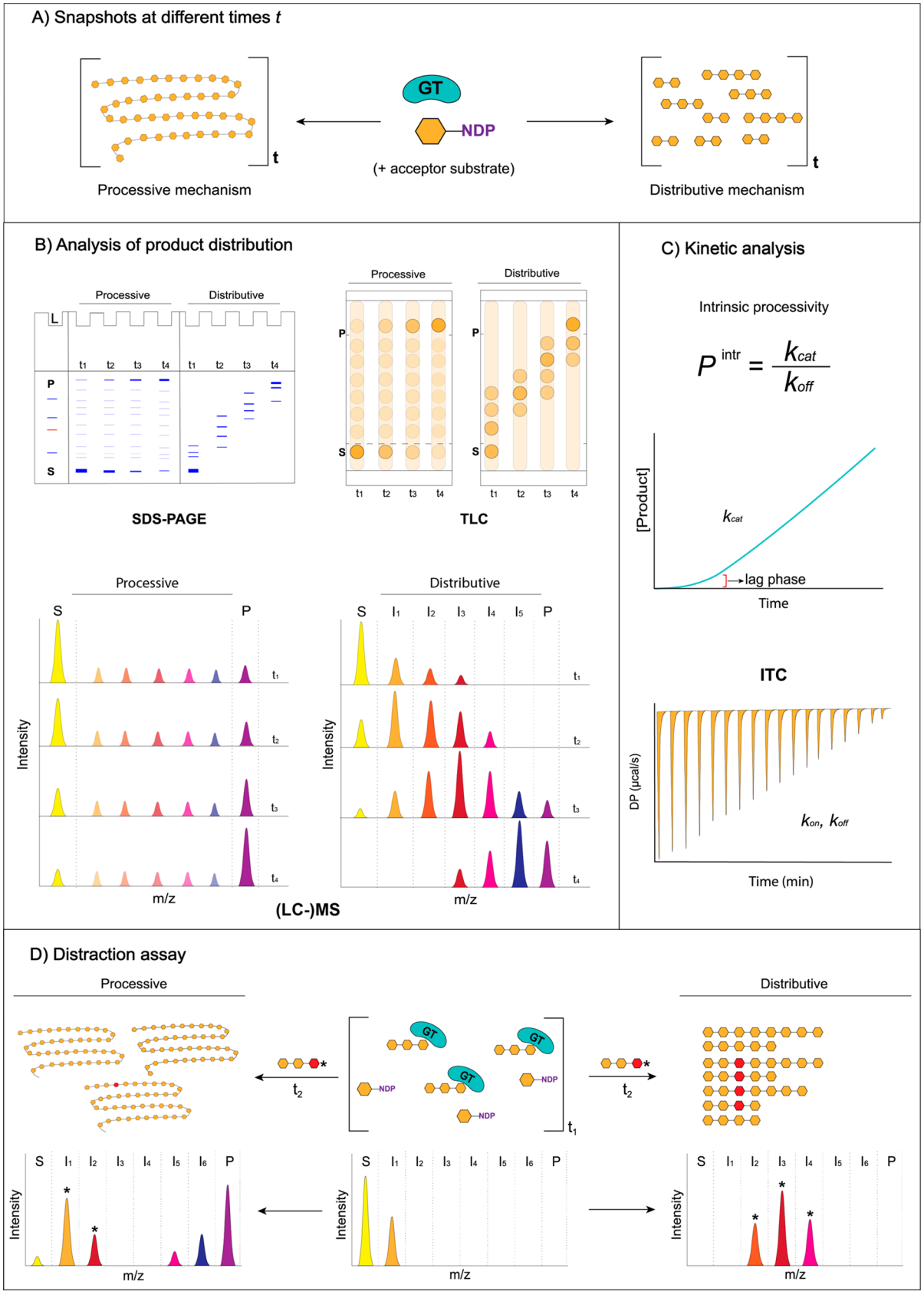

Figure 3. Overview of the methods most often used to study processivity. (A) Theoretical outcomes of the reaction at times *t* when the mechanism is processive (left) or distributive (right). (B) Analysis of product distributions by SDS-PAGE, TLC and (LC-)MS. (C) Kinetic analysis of the reaction mechanism. (D) Experimental setup of a distraction assay, with the potential product profiles.

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PROCESSIVITY IN POLYMERIZING GLYCOSYLTRANSFERASES

To produce the highly diverse pool of glycans present in all domains of life, unique GTs are needed to catalyze a specific reaction, and hundreds of known GTs have been classified into GT families based on protein sequence similarities (see www.cazy.org for an up-to-date overview of GT families).[23] The majority of mechanistic research has focused on the distinction between an "inverting" and "retaining" mechanism, which refers to the anomeric configuration of the new glycosidic bond in relation to the linkage in the donor.[24] Taking the glycosylation of wall teichoic acids as an example (Figure 2), in an inverting mechanism the anomeric configuration of the carbohydrate in the nucleotide-activated donor (e.g. UDP-α-GlcNAc) is opposite to the configuration in the product (e.g., β-GlcNAc synthesis by TarS). In contrast, the anomeric linkage in the donor and product are of the same configuration in a retaining mechanism (α-GlcNAc synthesis by TarM). Another interesting feature of GTs that produce polysaccharides is the direction of elongation; new carbohydrate units may be added to the reducing end (i.e., to the anomeric center of the acceptor substrate), or to the nonreducing end of the growing chain. Independent of the direction of elongation, the growing chain may be linked at the reducing end to the nucleotide-diphosphate moiety from the donor (as in hyaluronan synthase)[4] or a lipid moiety (as in peptidoglycan transferase, *vide infra*).

A prominent example of a processive glycosyltransferase is cellulose synthase. Cellulose is the major constituent of the plant cell wall, and also several bacterial species synthesize cellulose as part of their biofilm formation. It is a linear polysaccharide that consists of β-1 → 4 linked glucopyranosides, and lengths of up to 15 000 glucose units have been reported in plants.[25] Cellulose is synthesized by a membrane-integrated cellulose synthase complex (CeS), which varies greatly between species but all share a conserved catalytic subunit. CeS is an inverting GT that is classified in the GT2 family, and it synthesizes cellulose by adding Glc units from the UDP-Glc donor to the nonreducing end of the growing substrate.[26] Glycosylation is efficiently coupled to translocation across the membrane, so that the growing chain is immediately exported.[27] Processivity is inferred from kinetic analysis and the tight binding of the enzyme with the acceptor substrate.[28] More details on the mechanism of the cellulose synthase enzymes can be found in a recent review.[29]

Next to the investigations into the processivity in cellulose synthesis, processive mechanisms have been established for the membrane-integrated synthases producing chitin[30] and hyaluronan.[31,32] The associated enzymes all belong to GT family 2 of inverting β-glycosyltransferases, and they couple processive polymerization at the membrane interface with translocation to the extracellular space.[26] More recently, with the increasing awareness of the involvement of bacterial polysaccharides in pathogenicity and virulence, the mechanisms of the GTs responsible for the production of these bacterial structures have received increasing attention. As shown below, these reports reveal that processivity is a widespread mechanistic feature that occurs across different GT families.

Peptidoglycan. Peptidoglycan (PG, structure A in Figure 2) is a dense cross-linked layer of polysaccharides present in the cell wall of all bacteria. PG is initially constructed as a linear polymer of alternating GlcNAc (NAG) and MurNAc (NAM) residues, which are in a later stage cross-linked by transpeptidases. The linear PG is formed by the action of peptidoglycan transferase (PG transferase, also called transglycosylase, GT family 51) in the extracellular space that transfers an undecaprenyl-pyrophosphate-linked NAG-NAM dimer (Lipid II) to the reducing end of the growing lipid-linked NAG-NAM repeat.[34] PG chain length varies tremendously between bacterial species, and lengths of 3 to 250 disaccharide units have been reported,[33] which seem to be a result of the nature of the PG transferase enzyme instead of the enzyme to acceptor ratio.[34] PG transferase from *Aquifex aeolicus* was shown to operate through a processive mechanism, as under steady-state conditions no small NAG-NAM repeats were observed (gel-electrophoresis assay), but instead, a ladder of up to 40 NAG-NAM repeats was visible.[35] This *in vitro* experiment was complemented by structural information that suggests that the PG transferase contains a flexible helical "flap" that closes upon catalysis and retains the growing PG chain in the active site. Moreover, a donor and acceptor site cooperativity has been described, because the enzyme flexibility is greatly reduced when both donor and acceptor substrate are bound, which is proposed to aid in the processive mechanism.[36] The determination of structural features in this case helps to understand the determinants of processivity: the PG chain is locked in the active site allowing the next repeating unit to be added, instead of product release. Also the characteristic lag-phase was observed in a fluorescent kinetic assay, which disappeared when the concentration of the first catalytic product had increased.[37] Alternatively, the lag-phase also disappeared when synthetic Lipid IV (a Lipid II dimer) was directly used as the donor, bypassing the first catalytic step.[38] Together, knowledge of the structural elements that retain the growing chain in the active site and the preference for more elongated substrate provides strong support for processive behavior. Recently, two new types of PG transferases, called RodA and FtsW, were reported that belong to the Shape, Elongation, Division, and Sporulation (SEDS)-family of proteins.[39−41] Initial hints at the catalytic mechanism have started to appear (i.e., reducing-end elongation),[42] so it will be interesting to understand the degree of processivity of these enzymes. Because PG transferase processivity has been implicated as a mechanism to ensure bacterial cell straightening after deformations caused by external stress, these enzymes have a direct impact on bacterial fitness and antibiotic resistance.[43,44]

Wall Teichoic Acids. Wall teichoic acids (WTAs, structure C in Figure 2) in *Staphylococcus aureus* consist of 40−60 repeats of polyribitol phosphate, and are heavily decorated with *O*-GlcNAc residues, which appear to be important for antibiotic resistance and virulence.[45] The GlcNAc units are attached by the GT enzymes TarM and TarS through an α- and β-linkage, respectively (Figure 2). Using structural studies of both GTs in complex with their substrates and products, it was deduced that TarM and TarS form trimers.[46,47] For the retaining enzyme TarM, the trimerization did not seem to have an impact on processivity, as the WT and a trimerization-impaired mutant (G117R) showed equally high intrinsic processivity (P^{intr} 11 700 ± 1200 and 12 800 ± 1100, respectively, obtained from measuring k_{on} and k_{off} using biolayer interferometry).[46] The authors propose that a positively charged groove in the acceptor-binding domain is important for binding the negatively charged acceptor substrate and facilitate its sliding during processive GlcNAc transfer. Interestingly, the trimerization is more important for the inverting enzyme TarS, as the wild-type showed a P^{intr} of 2400 ± 260, compared with the truncated enzyme lacking the trimerization domain, which showed a P^{intr} of 133 ± 14, suggesting that the trimerization induced a

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processive mechanism.[47] This difference between TarM and TarS is an intriguing discovery, and more research may shed light on the contribution of trimerization to processivity. Processivity may arise from the trimeric enzyme that modifies multiple substrate molecules simultaneously or from the geometry of the trimer that helps to thread the growing substrate. Since the kinetic experiments were performed through analysis of the amount of UDP released instead of identification of product formed, the questions remain whether TarM and TarS have a certain spatial preference of adding GlcNAc residues and the precise direction of catalysis for the polyribitol phosphate acceptor.

Capsular Polysaccharides. Bacterial capsular polysaccharides (CPS) are highly diverse long-chain structures with a repetitive nature that vary greatly between bacterial species and serotypes (structures D−G in Figure 2).[48,49] They form a dense capsule around many pathogenic bacteria and are both a first line of defense and a virulence factor.

The CPS of *Streptococcus pneumonia* 3 is composed of cellubiuronic acid (structure D in Figure 2), a polymer containing [3-β-D-GlcA-(1 → 4)-β-D-Glc-(1 →] disaccharide repeats, reaching sizes of 50−1150 kDa. The responsible enzyme cellubiuronan synthase (Cps3S, or Cap3B) contains the signature motif QXXRW that is also found in the polymerases that produce cellulose, chitin, and hyaluronan, which may be a first indication of potential processivity.[17] Cellubiuronic acid is synthesized by the addition of carbohydrate residues to the nonreducing end in a processive mechanism.[50] Sps3S kinetics show a distinct lag phase during which the enzyme is assumed to produce a lipid-linked oligosaccharide of ∼4 dimer repeats,[51] followed by the full processive state in which rapid elongation is accomplished. Initial hints for this processivity were obtained through a pulse-chase experiment, in which an *in vitro* reaction was started with isotopically labeled carbohydrate donor, followed by a pulse of nonlabeled donor and a chase for 5 and 20 min.[50] The majority of labeled carbohydrates were incorporated in the longer polymers, indicating a tight association between the enzyme and the elongating product. When the reaction temperature was increased to 54 °C, the fraction of polysaccharide was greatly reduced while the contribution of shorter oligosaccharides had increased, presumably because the rate of premature dissociation was higher at elevated temperatures.[51] These experiments strongly suggest a preference for elongated substrate and a tight association, which are hallmark characteristics of a processive enzyme. Structural information will complement these observations and may offer an explanation for the apparent processive behavior.

The CPS structures of *Neisseria meningitidis* are negatively charged, and in the case of the serogroups A and X, this is caused by a phosphodiester moiety linking the carbohydrates (structures E and F in Figure 2), resulting in the polymers CPSA (poly-α-1 → 6-ManNAc-1-OPO_3^-) and CPSX (poly-β-1 → 4-GlcNAc-1-OPO_3^-), respectively, which share a common glycolipid membrane anchor with polysialic acid (*vide infra*).[52] The biosynthesis of these polymers has received widespread interest because of their antigenicity, which subsequently led to their use in the development of synthetic vaccines.[53−55] CPSX is synthesized by the GlcNAc-transferase CsxA, which was shown to act in a processive manner. When exposed to increasing ratios of UDP-sugar donor and a short oligosaccharide acceptor, there was a switch from the production of short to long products (gel electrophoresis).[55] This suggests an optimal donor/acceptor ratio at which the tightly associated CsxA is able to produce longer products. This could reflect optimal conditions to ensure processivity *in vivo*, with access to CPS of defined lengths as a result. In contrast, an increasing donor to acceptor ratio for CsaB, responsible for synthesizing CPSA, resulted in a stepwise increase in product size, suggesting it uses a distributive mechanism. When compared with CsaB, processive CsxA harbors an additional 98 amino acid residues at the C-terminus, which proved to be essential for processivity, since the removal of this section turned CsxA into a distributive enzyme, as further illustrated by HPLC-AEC elution profiles of the products.[55] It was suggested that the C-terminal section of CsxA acts as a product-binding domain, as a complex with the CPSX product was resistant to proteolysis.[55] Similarly to other examples, this structural motif contributes to processivity by retaining the product near the active site and allowing the enzyme to perform the next round of catalysis. Interestingly, by also removing the N-terminal section involved in oligomerization of CsxA monomers, a narrow product distribution was obtained, which may find application in vaccine development.

Polysialic acid (polySia, structure G in Figure 2) chains with lengths of up to 400 sialic acid residues are important constituents of the CPS of certain Gram-negative bacteria and serotypes, including *Neisseria meningitidis*, *Escherichia coli*, *Moraxella nonliquefaciens*, and *Mannheimia haemolytica* (*Mh*). Bacterial polysialyltransferases (PSTs) are classified in GT family 38 and are able to construct homopolymers of α-2 → 8-linked (as in *E. coli* K1 and *N. meningitidis* serogoup B) or α-2 → 9-linked sialic acids (as in *N. meningitides* serogroup C), or heteropolymers of alternating α-2 → 8/α-2 → 9-linked residues, all on a β-Kdo-lipid precursor.[52] Interestingly, mammalian PSTs belong to GT family 29 and solely produce homopolymers of α-2 → 8-linked sialic acids linked to proteins, such as neural cell adhesion molecule (NCAM). Because these polySia chains are central carbohydrates in eukaryotic cell−cell signaling in the brain and during embryonic development, the bacterial polySia capsules successfully evade the immune system. On the one hand, this molecular mimicry hampered the application of vaccines generated against these bacterial polySia capsules, but it was successfully exploited to improve the pharmacokinetic properties of human therapeutic proteins by decorating them with poly-α-2 → 8-Sia chains through the action of bacterial PSTs.[56] A comparison of structural data of *Mh*PST and a mammalian PST (ST8SiaIII) revealed that, although both enzymes create the same type of α-2 → 8-linked polySia chains, the enzymes share no common structural features.[57] In *Neisseria meningitidis* serogroups B (*NmB*) and C (*NmC*) and *Escherichia coli* K1, the polySia in the capsule is essential for virulence, making the polySia-producing enzymes a potential antimicrobial target. The structural differences between the bacterial and mammalians PSTs, leading to higher substrate promiscuity for the first, may aid in selective inhibitor development.[58]

Contradicting reports exist on the degree of processivity displayed by the PSTs, which may be explained by their different origins or the different conditions used in the experiments. The latter reason is especially apparent for the bacterial PSTs, which are membrane-bound enzymes. Reaction conditions, including the method to solubilize the membrane-bound enzyme and acceptor lipid, seem to have a major impact on the reaction kinetics and product length distributions. When a soluble truncated *Nm*BPST construct was used to elongate a trisialoganglioside acceptor, a product pattern consistent with a distributive mechanism was observed by HPLC analysis.[59] A

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similar conclusion was derived for the *NmC* homologue upon successful distraction of a running reaction with an unnatural acceptor.[60] Alternatively, in an *in vivo* experiment, in which the expression of *Ec*PST was induced in the absence of endogenous PST activity, a processive mechanism was postulated on the basis of the product distribution (visualized with TLC analysis).[61] Moreover, when *EcK92*PST was solubilized in native membranes, the shorter product lengths (DP < 10) suggest a distributive mechanism, whereas the larger polySia chains (DP > 12) are rapidly elongated to form the full-length poly-Sia.[62] This latter finding suggests an increasing product affinity upon elongation that induces an increase in the degree of processivity. Bacterial PSTs are postulated to be part of a large biosynthetic complex *in vivo*, also containing translocation machinery that may assist in exporting the growing polysaccharide. Since processivity has been coupled to translocation in other well-studied systems including cellulose synthesis,[27] the processive mechanism may become apparent in more *in vivo*-like experimental systems.

Information on the degree of processivity can also be obtained by structural analyses and binding studies. STD-NMR studies with full-length *NmB*PST suggested an extended acceptor binding site that can accommodate up to six Sia residues.[63] In addition, crystallographic analysis of the *Mh*PST reveals a deep electropositive groove in between the two Rossmann-fold domains that binds the acceptor mimetic fondaparinux in the open state.[57] It is postulated that the active site closes during catalytic activity to retain the polyanionic growing chain, suggesting a processive mechanism. The importance of acceptor binding for the degree of processivity was highlighted in a seminal contribution by the group of Gerardy-Schahn.[64] Using a neutral genetic drift approach to quickly engineer *NmB*PST variants, a single amino acid in the acceptor-binding domain was identified that could toggle the mechanism between processive and distributive (product profiles analyzed by HPLC). Moreover, a number of basic residues were identified in the acceptor-binding site that were individually contributing to the increased processivity, suggesting their importance in binding the growing polySia chain. This is a great example of how in-depth structural studies contribute to understanding the underlying reasons for processivity and allow to manipulate and tune the mechanism of the enzyme, depending on the application in mind. Moreover, it highlights the importance of studying and exploiting processivity and its determinants as a way to expand the enzymatic toolbox.

Lipopolysaccharide O-Antigens. O-Antigens are long polysaccharide motifs that are covalently attached to the lipopolysaccharide (LPS) molecules in most Gram-negative bacteria (structure H and I in Figure 2).[65] They consist of one or multiple types of monosaccharides and provide a great structural diversity to the extracellular glycocalyx of bacteria. There are three different pathways through which the O-antigens may be biosynthesized, which are primarily characterized by the translocation machinery: the Wzy-dependent pathway, the synthase-dependent pathway, and the ATP-binding cassette (ABC)-transporter-dependent pathway.[66] The Wzy polymerase is found to act in a nonprocessive (distributive) manner, while the other two generally are involved in processive elongation.[67] O-antigens are generally synthesized on the cytosolic side of the inner membrane, as is the Lipid A core, after which they are ligated in the periplasm. It has been suggested that the polymerization of O-antigen is halted by this ligation event, leading to a modal distribution of O-antigen lengths specific to bacterial species and even serotypes.

Salmonella enterica serogroup O:54 produces an O-antigen that consists of ManNAc residues alternately connected through β-1 → 3 and β-1 → 4 linkages in a synthase-dependent pathway (structure H in Figure 2). The O-antigen synthesis starts by the addition of a single ManNAc unit to an undecaprenol-pyrophosphate-GlcNAc acceptor by the monofunctional WbbE (RfbA). Next, the acceptor is elongated at the nonreducing end by WbbF (RfbB, GT family 2) using a processive mechanism, which is proposed by sequence similarity to other processive transferases.[68,69] As synthases are integral membrane proteins, WbbF is predicted to simultaneously perform glycosyl transfer and translocation of the growing chain, as a mechanism to support processivity. Currently, molecular details of the structural determinants underlying this mechanism remain elusive.

An example of processive O-antigen synthesis through the ABC-transporter-dependent pathway is the construction of D-galactan I from *Klebsiella pneumoniae*, which is composed of [3-β-D-Gal*f*-(1 → 3)-β-D-Gal*p*-(1 →] disaccharide repeats (structure I in Figure 2). The Und-PP-GlcNAc acceptor is first elongated with Gal*p* and Gal*f* by bifunctional WbbO and subsequently elongated by both WbbO and WbbM at the nonreducing end of the lipid-linked acceptor substrate using nucleotide-activated donors. Kinetic experiments suggest that both enzymes are involved in the processive synthesis of D-galactan I, as no products of intermediate lengths are observed (judged by the level of incorporation of radioactivity).[70] Further studies that generate a more detailed picture (e.g., product profiles, kinetic, and binding studies) are needed to provide solid proof of processivity and unravel the enzymatic determinants that result in this processive behavior.

Mycobacterial (Arabino)galactan. The mycobacterial cell wall contains a linear polymer of 20−40 galactofuranose (Gal*f*) units termed the galactan (structure K in Figure 2). Because of its important role in cell wall maintenance and survival of *Mycobacteria*, the biosynthesis of (arabino)galactan is of high interest.[71] The bifunctional enzyme GalfT2 uses a single active site[72] to produce galactan with alternating β-1 → 5 and β-1 → 6 linkages by adding Gal*f* units (from UDP-Gal*f*) to the nonreducing end of a decaprenyl-pyrophosphate-linked GlcNAc-Rha-Gal*f*-Gal*f* as the acceptor tetrasaccharide.[73,74] Kiessling and co-workers obtained initial hints that GalfT2 is a processive enzyme by the remaining presence of the lipid-linked acceptor while products with 3−27 added Gal*f* units were produced.[75] Moreover, when the reaction was performed with the glycolipid acceptor in 1000-fold excess over the enzyme, the elongated products were observed even at the earliest time points, suggesting that the enzyme stays associated with the product to perform multiple rounds of catalysis. Chemical synthesis gave access to a selection of lipids varying in length, and only the longer lipids showed processive elongation *in vitro*, suggesting a high affinity for the lipid moiety in a tethering mechanism.[75] Additionally, X-ray crystallography studies revealed that GalfT2 forms a homotetramer that associates with the membrane, so that the lipid acceptor may freely bind to and dissociate from both the enzyme and lipid bilayer.[76] Subsequently, processivity was further proven by a distraction assay under single-hit conditions (i.e., a large acceptor substrate to enzyme ratio).[77] The GalfT2 reaction was started in the presence of the regular acceptor substrate, followed by the addition of an isotopically labeled acceptor. After a second period, the product distribution was analyzed by MALDI-TOF, and this analysis revealed that most isotopically labeled acceptor

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was elongated with only a few Gal*f* units, while the long Gal*f* polymers were attached to the regular acceptor. The effort to elucidate the mechanism of GalfT2 is an exemplary case where multiple methods are used to unequivocally conclude processivity: product distribution studies and single-hit and distraction assays revealed continuous association of the enzyme and substrate, while structural studies showed tethering and membrane association.

SEMIPROCESSIVITY IN POLYMERIZING GLYCOSYLTRANSFERASES

An unusual class of processive GTs is formed by the enzymes that catalyze a limited number of glycosyl transfers processively, before they release the product. Such enzymes are identified as semiprocessive, and they may display both processive and distributive characteristics. For example, in *Campylobacter jejuni* N-glycan synthesis, a trisaccharide is assembled on an undecaprenol-diphosphate anchor in the membrane and subsequently elongated by the action of PglH (Figure 4A).[78]

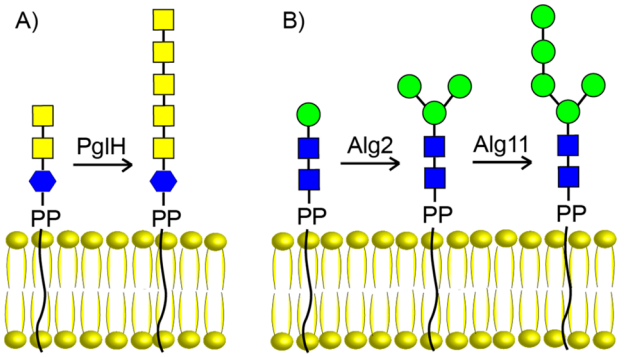

Figure 4. Schematic overview of semiprocessive PglH (A) and Alg2 and Alg11 (B).

This enzyme transfers three *N*-acetylgalactosamine units sequentially without releasing the growing substrate in a semiprocessive manner, as determined by radiolabeled product profiles (HPLC). Kinetic studies indicated that the first and second GalNAc addition are fast and that the binding affinity of PglH for the glyco-products increases with every GalNAc addition. Recent structural studies of PglH bound to a lipid-linked product analogue revealed that after the transfer of three GalNAc residues, the pyrophosphate moiety at the reducing end is pushed to make contacts with positively charged residues in the potential "ruler helix".[79] This interaction is suggested to inhibit subsequent sliding of the product which would be needed for the transfer of additional GalNAc residues, and as a result, the product is released after three GalNAc additions. In this case, it is apparent that the increasing affinity for modified substrate is a determinant of processivity, whereas the structural control of trisaccharide release is a distributive feature. A combination of these traits results in semiprocessivity.

In the *Mycobacterial* galactan synthesis, the processive action of GlfT2 (*vide supra*) is preceded by the sequential addition of two Gal*f* residues to decaprenyl-pyrophosphate-linked GlcNAc-Rha disaccharide by GlfT1 to create the tetrasaccharide acceptor substrate.[74,80] *In vitro* experiments suggest the processive addition of two Gal*f* units, since the single addition-product is not observed (MALDI-TOF), but more experiments are needed to confirm processivity of GlfT1.[81] Other bacterial GTs that transfer residues iteratively include WaaA (formerly KdtA) in *E. coli*, which is involved in the transfer of two Kdo residues in the synthesis of Lipid A.[82] Interestingly, WaaA homologues in *Chlamydia* species can be also tri- and tetrafunctional, whereas homologues have been identified that just transfer a single Kdo residue.[65] It is interesting to investigate whether these Kdo residues are added using a processive or distributive mechanism and how length control is established.

Eukaryotes also harbor semiprocessive GTs, such as Alg2 and Alg11 from *Saccharomyces cerevisiae*, which are both involved in *N*-glycan synthesis on the cytosolic face of the ER (Figure 4B). Interestingly, Alg2 is a bifunctional enzyme that attaches α-1 → 3 and α-1 → 6 linked mannosides consecutively to the dolichol-pyrophosphate-GlcNAc$_2$Man acceptor, as identified by HPLC analysis of the glycan products.[83,84] Subsequently, this lipid-linked pentasaccharide is further elongated by the sequential addition of two α-1 → 2 mannosides by Alg11.[84,85] Whereas intermediate products (i.e., after a single mannosylation event) have not been observed for both enzymes, the definite proof for a processive mechanism has not yet been provided.

PROCESSIVITY IN PROTEIN GLYCOSYLTRANSFERASES

Selected families of eukaryotic and bacterial proteins are found to be heavily modified with various sugars resulting in a hyperglycosylated protein surface. In bacteria, these proteins include adhesin proteins, autotransporters, pili, and flagella, which are involved in pathogenic traits such as adhesion to host cells and provide motility, physical insertion in the mucus layer, and resistance to shear stress. In the examples known to date, hyperglycosylation is achieved via the action of a single or multiple cytoplasmic glycosyltransferases that use nucleotide-activated monosaccharides to directly modify their protein substrates. Intriguingly, protein hyperglycosylation may also be performed in a processive manner and identifying the degree of processivity will be important to develop strategies to inhibit this hyperglycosylation.

Well-known examples of hyperglycosylated adhesin proteins are the serine-rich repeat proteins (SRRPs) in Gram-positive *Streptococcal*, *Staphylococcal*, and *Lactobacilli* species, where SRRPs are responsible for adhesion and/or biofilm formation. Examples of these hyperglycosylated adhesion virulence factors include Srr1 from *Streptococcus agalactiae*,[86,87] Fap1 from *Streptococcus parasanguinis*,[88] PsrP from *Pneumococcal* species,[89,90] GspB (structure B in Figure 2) and Hsa from *Streptococcus gordonii*, and Srap from *Staphylococcus aureus*.[88] The first step of multisite O-GlcNAcylation in the Ser/Thr-rich domain is catalyzed by the GtfA/GtfB enzyme complex (also termed Gtf1/Gtf2), and it provides the basis for further modification and glycan diversification. GtfA is a glycosyltransferase (GT family 4) and GtfB is an associated chaperone responsible for the stability of the complex and it conveys affinity for the protein substrate and glyco-intermediates.[91,92] Recently, the first crystal structure of the GtfA/GtfB complex was published, together with data from kinetic and binding experiments.[92] An *in vitro* activity assay of the GtfA/GtfB complex from *Streptoccocus gordonii* with truncated versions of the GspB adhesin substrate showed a distinct two-step mechanism of glycosylation (gel electrophoresis). In the first "fast" phase, an accumulation of glyco-intermediates of varying degrees of O-GlcNAc-modification was observed (gel electrophoresis), which were transformed into fully glycosylated products in the second "slow" phase, suggesting a distributive mechanism. Moreover, when the reaction was diluted to prevent

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reassociation (single-hit conditions), the GtfA/GtfB reaction did not proceed. It will be interesting to investigate the mechanistic features of the functional homologues of GtfA/GtfB in other species.

Hyperglycosylation has also been identified in ClfA (clumping factor A) in *Staphylococcus aureus*, which is one of the ESKAPE pathogens and a difficult Gram-positive pathogen to combat due to the complex network of virulence factors. Among the identified surface proteins of *Staphylococcus aureus*, ClfA is heavily modified with O-GlcNAc moieties, which mediate attachment to host cells and evasion from the host immune system.[93] The first step of hyperglycosylation is performed by SdgB, which adds GlcNAc units to the serine residues in serine-aspartate dipeptide-repeats (SDR). Next, the majority of O-GlcNAc moieties is decorated by an additional GlcNAc by the action of SdgA transferase, resulting in a disaccharide motif. Mass analysis of *in vitro* glycosylation reactions on the purified SDR-region (expressed as soluble construct) revealed that 59 GlcNAc units are added by SdgB, followed by an additional 47 units by SdgA, with no detectable intermediate products. This may suggest some degree of processivity, but a detailed kinetic investigation is warranted to provide additional proof.

One of the early examples of extensive protein asparagine-glycosylation in Gram-negative bacteria was identified in nontypeable *Haemophilus influenzae* (NTHi), where HMWA (high-molecular weight) adhesin proteins were found to be highly N-glycosylated with single glucose units by their cognate HMW1C glycosyltransferase.[94,95] Preliminary results from our group hint at some degree of processivity for HMW1C and its structural and functional homologue from *Actinobacillus pleuropneumoniae*. By utilizing intact protein mass spectrometry to monitor progression of glycosylation over time, complemented with a distraction assay, affinity studies and proteomics, we were able to visualize the kinetic behavior of *Ap*/*Hi*-HMW1C-catalyzed hyperglucosylation. Our data suggests processive behavior for these enzymes at certain stages of the reaction, and we are currently investigating the rationale behind these findings.

Gram-negative bacteria also may display another family of adhesins called the autotransporter proteins. They possess a C-terminal beta-barrel domain and a variable N-terminal passenger domain that allow them to perform self-transport as well as adhesion functions, respectively.[96] One of the most prominent examples is the family of self-associating autotransporters (SAAT) from *E. coli* species. Members of this group include AIDA-I (adhesin involved in diffused adherence), the TibA autotransporter (structure J in Figure 2), and aggregation factor Ag43. All three proteins are modified with heptosides on multiple Ser/Thr sites by the action of associated transferases (Aah[96] and TibC[97]), which are also functionally interchangeable.[98] Similar to other O-glycosylation machinery, these transferases lack a conserved amino acid recognition sequence but instead rely on structural motifs for recognition, as was discovered in the studies of the Aah transferase.[99] Interestingly, both Aah and TibC belong to the dodecameric iron-containing family of enzymes. It was shown that the architecture in which the 12 subunits form two stacked six-membered rings is important for its mechanism,[97] as only the inside of the ring was performing catalysis, modifying six molecules of substrate simultaneously in a so-called "screw-propelling" mechanism. The mechanism of TibA hyperheptosylation was therefore hypothesized to include processive features. This is also in line with the "crowding reasoning" which suggests that processive modifications are preferred over distributive ones in confined environments due to subdiffusion.[100] However, kinetic experiments are needed to further validate the postulated hypothesis of TibC/Aah processivity. Other examples of hyperglycosylated adhesins are identified in enterotoxigenic *Escherichia coli* (EtpA, TPS adhesin),[80] *Kingella kingae* (KnH autoransporter), and *Aggregatibacter aphrophilus* (EmaA autotransporter), but the mechanisms of the responsible GTs remain elusive.

As apparent from the examples above, the first data on processivity in protein glycosyltransferases is currently starting to appear. Processivity is a complex mechanistic trait to investigate, especially in glycoproteins that are larger in size, and it requires thorough investigation using different methods to draw solid conclusions. An example of such a thorough approach in protein glycosylation is the research on the distributive character of the mammalian O-GlcNAc transferase.[101] In examples that currently lack concrete evidence, additional steps can be taken to solidify the conclusions. For instance, a distraction assay with (labeled) protein substrate can be used to prove processivity of the *S. aureus* SdgB and SdgA glycosyltransferases. In the case of TibC/Aah, monitoring the reaction for intermediates and determining the kinetic parameters will further add to existing structural evidence of processivity. These and other experiments will be important to elucidate the mechanism of protein hyperglycosylation in pathogenic bacteria.

PROCESSIVITY AND INHIBITOR DESIGN

Knowledge of specific mechanistic features, such as the catalytic residues, structure of the transition state, and metal ion involvement, is generally a good starting point for rational drug design.[22] Additionally, determining the enzyme structure with substrate or inhibitor bound is useful to avoid targeting eukaryotic GTs in the same fold family. Especially when the enzyme acts on a unique bacteria-specific carbohydrate residue, knowledge of its binding may provide handles for specificity. The (degree of) processivity can become an additional asset, as processivity often stems from specific mechanistic or structural elements that can be exploited in enzyme-specific inhibitor design. In contrast, designing (competitive) inhibitors for processive enzymes may be complicated by the high affinity for their substrates and the presence of additional binding pockets and translocation machinery.

To the best of our knowledge, a few examples exist where determinants of processivity are exploited in inhibitor design. In the case of GalfT2 from *Mycobacteria*, processivity inspired the generation of disaccharide-like inhibitors. The enzyme prefers elongating a Gal*f*-Gal*f* motif, and these disaccharide analogues were shown to inhibit moderately at mM concentrations.[102] As a Gal*f*-trisaccharide is an even better acceptor,[103] increasing the size of the inhibitors to trisaccharide analogues may increase the inhibitory potency. To inhibit peptidoglycan synthesis that occurs at the membrane interface, lipid-linked GlcNAc-MurNAc derivatives were produced as inhibitors for peptidoglycan transferase,[104] These Lipid II analogues showed good inhibition of PG transferase *in vitro* and are postulated to be good binders to both the donor and acceptor binding sites. Furthermore, in experiments inspired by the hydrophobic acceptor-binding groove in bacterial cellulose synthase BcsA, donor analogues that are extended on the uridine moiety with aromatic residues gave decent IC_{50} values.[105] These examples serve to showcase that understanding the mechanistic character-

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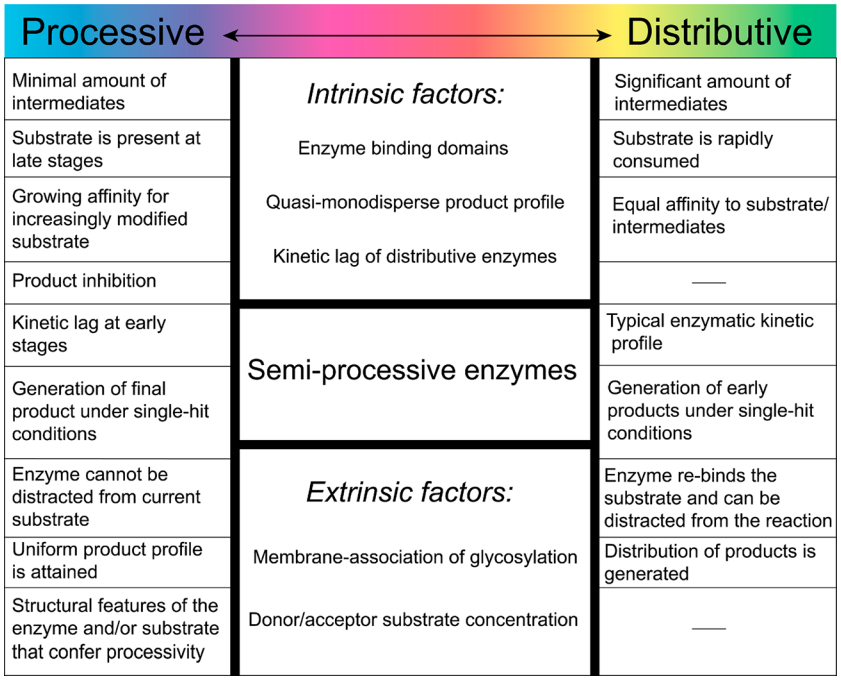

Figure 5. Integrative table of the characteristic features of processivity and distributivity on both ends of the continuum, with examples of ambiguous characteristics in the middle.

istics and determinants of processivity may pave the way for specific processivity-targeted inhibitors.

CONCLUSIONS

Processivity is a mechanistic trait that is highly challenging to study. Analysis of the enzymatic reaction in terms of product distribution and kinetics and the use of experimental tricks like single-hit conditions or acceptor substrate distraction informs on the degree of processivity. One must recognize that processivity is not a singular enzymatic property, but rather a continuum of possibilities that are system-dependent. Strictly processive (or distributive) enzymes display an array of recognizable features (listed in Figure 5) that can be determined using methods described in this Review. However, researchers must exercise caution when interpreting experimental evidence, as in certain examples it might be misleading rather than informative. For instance, structural features such as the presence of large or tight substrate binding grooves, the presence of a kinetic lag phase, or a (quasi)-monodisperse product profile might suggest processivity, but can instead be intrinsic features of certain enzymes that are distributive in mechanism. For instance, the teichoic acid polymerase TagF has a large open active site that would allow continuous binding of CDP-glycerol-β-phosphate donor and release of CMP by-product during polymerization. However, it was found that the polymerization of glycerol phosphate is instead concentration-dependent and is catalyzed in a "non-continuous manner", i.e. distributively.[106] Furthermore, in the case of the class II hyaluronan synthase from *Pasteurella multocida*, a kinetic lag phase was observed at early stages of the reaction. However, by monitoring the polymerization reaction by TLC a ladder of products was observed, indicating a stepwise (distributive) mechanism.[107] Moreover, the quasi-monodisperse (processive-like) product profile only appeared when an oligosaccharide acceptor was used in the reaction.[108,109] Also external factors may play a role in the perceived processivity, as the lack of "native" conditions (e.g., the natural enzyme/substrate concentration or the presence of membrane translocation machinery) in *in vitro* reactions can influence the experiment tremendously. Mentioned in this Review are also semiprocessive enzymes that often possess a complex mechanistic profile with several distinctive features of both processivity and distributivity. Consequently, multiple carefully designed experiments are imperative to conclude the processivity (or lack thereof) in more challenging cases where conflicting evidence makes it difficult to place the enzyme under investigation on either side of the spectrum.

Many GTs suspected of processive catalysis still remain to be investigated. For instance, wall teichoic acid glycosylation in methicillin-resistant *Staphyloccocus aureus* (MRSA) is performed by TarP.[110] In analogy to TarM and TarS in the nonresistant strains, TarP may also display processive character. Additionally, in the case of protein glycosylation in MRSA, SDR (serine-aspartate repeats)-containing Pls (plasmin-sensitive protein) was recently discovered to be heavily glycosylated, which was important for (*in vitro*) biofilm formation.[111] Glycosylation is performed by the action of four glycosyltransferases, namely GtfC, GtfD, SdgA, and SdgB. As the latter two have been shown to act in a processive manner (*vide supra*), it is interesting to extrapolate these studies to GtfC/GtfD. In all, it is important to dedicate efforts toward understanding bacterial glycosyltransferase systems and mechanistic details as a first stone in the antibiotic firewall.

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Notes

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■ KEYWORDS

Processivity: the ability to remain associated with the growing substrate in between rounds of catalysis. This results in a predominant formation of highly modified products, while starting substrate may remain.

Distributivity: the fact that the enzyme dissociates after each catalytic reaction, resulting in a distribution of products.

Semiprocessive: a mechanistic trait that results in a limited number of catalytic reactions in a processive way, followed by enzyme dissociation.

Apparent processivity: the degree of processivity displayed by an enzyme under specific conditions, e.g. concentration and nature of the substrate, or the presence of membrane translocation machinery.

Intrinsic processivity: the distribution of probabilities that an enzyme will stay associated and catalyze another reaction or dissociate from the intermediate product.

Hyperglycosylation: addition of multiple carbohydrate moieties to the surface of a protein.

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