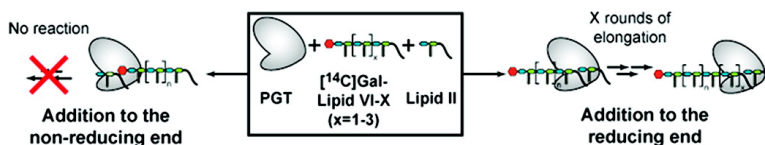


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## The Direction of Glycan Chain Elongation by Peptidoglycan Glycosyltransferases

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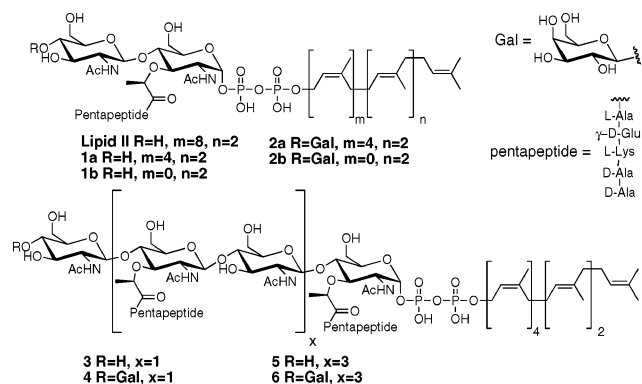
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Peptidoglycan glycosyltransferases (PGTs) are highly conserved bacterial enzymes that catalyze the polymerization of a disaccharide called Lipid II (Figure 1) to form the glycan strands of peptidoglycan. PGTs are regarded as desirable antibiotic targets because they are extracellular, they do not have eukaryotic counterparts, and they play an essential role in a validated therapeutic pathway.<sup>1</sup> Although their importance has been appreciated for decades, the mechanism of glycan chain polymerization is not yet understood. One aspect of the PGT reaction that is central to defining the mechanism is the direction of glycan chain elongation (Figure 2A).<sup>2</sup> Here, we describe an approach to test the direction of glycan chain growth. We have applied this strategy to four different PGTs from both Gram-negative and Gram-positive organisms, including two PGTs for which crystal structures were recently reported.<sup>3</sup> The results show that all these PGTs polymerize Lipid II by the addition of new disaccharide units to the anomeric (diphospholipid) end of the elongating polymer (called the “reducing end” by convention, although not a lactol).

Our strategy to determine the direction of glycan polymerization, illustrated in Figure 2B, relies on the synthesis of nascent peptidoglycan chains that are blocked at their nonreducing ends (Figure 2B). If elongation occurs by reaction at the reducing end of the growing polymer, PGTs should elongate these substrates in the presence of Lipid II (Figure 2B, right). If elongation occurs in the other direction, then these blocked oligomers will not be substrates for PGTs (Figure 2B, left).

To enable our strategy for probing the direction of chain elongation, we required a facile method to selectively modify the nonreducing ends of the nascent glycan chains. We chose to utilize  $\beta$ -1,4-galactosyltransferase (GalT), an enzyme that transfers galactose (Gal) from UDP-Gal to the C4-OH of terminal *N*-acetylglucosamine (GlcNAc) residues on a wide variety of molecules.<sup>4</sup> Incubation of synthetic Lipid II substrate analogue **1b** with GalT in the presence of UDP-Gal resulted in disappearance of **1b** and the concomitant appearance of a new compound with a mass consistent with the structure of the Gal-labeled analogue **2b** (expected and observed:  $m/z$  1491.6). To verify that Gal-labeling prevents reaction at the nonreducing end, we prepared heptaprenyl-<sup>[14C]</sup>Gal-Lipid II (**2a**) and <sup>[14C]</sup>Gal-Lipid IV (**4**) by treating heptaprenyl-Lipid II (**1a**)<sup>5a</sup> or heptaprenyl-Lipid IV (**3**)<sup>5b</sup> with UDP-<sup>[14C]</sup>Gal and GalT. No radiolabeled self-condensation products were detected when these substrate analogues were incubated with the PGT domain of *A. aeolicus* PBP1A. In the presence of Lipid II, however, the Gal-labeled substrates formed radiolabeled polymeric products (Supporting Information, Figures S1 and S2). These experiments demonstrate that Gal labeling of the nonreducing end of the Lipid II or Lipid IV substrates blocks this end from serving as a glycosyl acceptor.



**Figure 1.** Natural Lipid II as well Lipid II (**1**), Lipid IV (**3**), and Lipid VIII (**5**) substrate analogues and their Gal-labeled versions (**2**, **4**, and **6**).

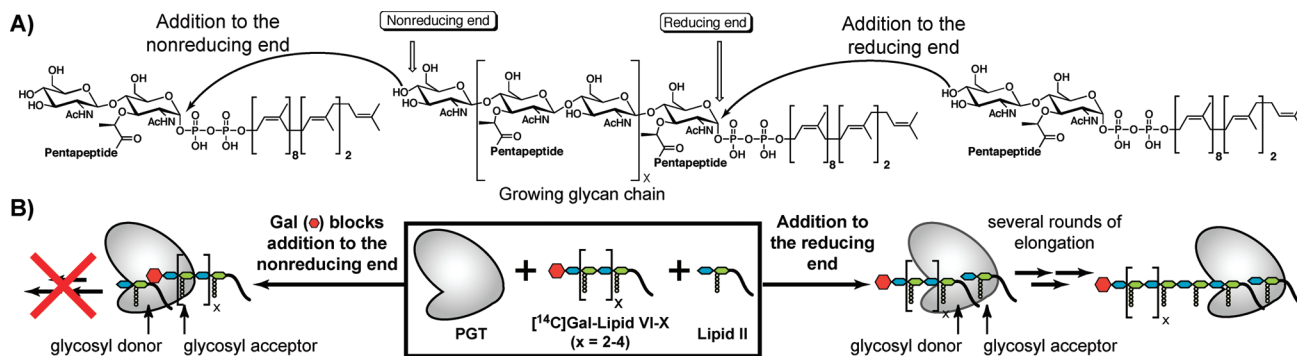
The experiments described above show that the Gal-labeled substrates can react at their reducing ends but not their nonreducing ends; however, they do not establish the direction of glycan chain polymerization because **1a** and **3** self-condense. Since these substrates are capable of reacting at *both* ends, their Gal-labeled analogues cannot be used to discriminate between the two possible mechanisms of elongation (see Figure S3). To establish the direction of chain elongation, we needed to identify peptidoglycan substrates that can be elongated by Lipid II but do *not* react with themselves. Nascent chains containing three or more disaccharide subunits meet this criterion.<sup>6</sup>

To synthesize the longer Gal-blocked substrates (Figure 3A) required to test the direction of chain elongation, we incubated compound **3** with a high concentration of *E. coli* PBP1A. Under these conditions, **3** reacted to give a mixture of products in which the major product, identified by its exact mass, was the octasaccharide Lipid VIII (**5**, Figure 1; Table S1). The nonreducing ends of the oligomer mixture were derivatized with <sup>[14C]</sup>Gal by treatment with GalT. The resulting blocked oligomers were purified by C18 chromatography and analyzed by gel electrophoresis (Figure 3B, lane 2).<sup>7</sup> Assignment of oligomer sizes was based on exact mass measurements of the Lipid IV polymerization reactions (Table S1) and on the electrophoretic mobilities of Gal-labeled oligomers (Figure S2).

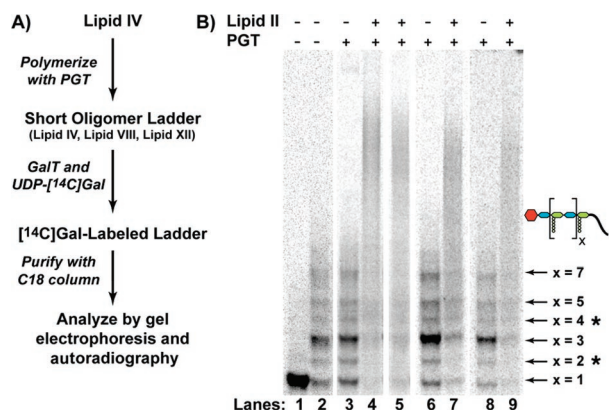
To assess the direction of chain elongation, the Gal-oligomers were incubated with four PGTs, including *E. coli* PBP1A, *E. coli* PBP1B, *A. aeolicus* PBP1A, and *S. aureus* PBP2,<sup>3a,5b,8</sup> in the presence and absence of Lipid II. The reactions were analyzed using a recently developed gel electrophoresis assay.<sup>3a,6</sup> In the absence of Lipid II, the distribution of oligomers was unchanged (compare lanes 3, 6, and 8 to control lane 2, Figure 3B). In the presence of Lipid II, the oligomers disappeared and a smear of radioactivity appeared higher up in the gel (lanes 4, 5, 7, and 9, Figure 3B), indicating that longer peptidoglycan products were formed. Since

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**Figure 2.** Models for the direction of glycan polymerization: (A) scheme showing that a glycan strand can be extended by adding new units to the nonreducing end (left) or to the reducing end (right); (B) scheme showing our strategy to test the direction of chain elongation. If the PGT is incubated with Lipid II and a polymer substrate blocked at the nonreducing end with  $^{14}\text{C}$ Gal, then the polymer will only be extended if new units add to the reducing end.



**Figure 3.** Gel electrophoresis assays to determine the direction of elongation: (A) scheme for the synthesis of the Gal-oligomer substrates; (B) gel electrophoresis of PGT reaction mixtures. Each lane contains either  $^{14}\text{C}$ -Gal-Lipid IV (lane 1) or the  $^{14}\text{C}$ -Gal-oligomer mix (lanes 2–9) incubated in the presence or absence of PGT and Lipid II. The PGTs investigated include *E. coli* PBP1A (lanes 3, 4), *E. coli* PBP1B (lane 5), *A. aeolicus* PBP1A (lanes 6, 7), and *S. aureus* PBP2 (lane 8, 9). The identities of the Gal-labeled oligomers, as assessed by electrophoretic mobilities (Figure S2)<sup>6</sup> and MS analysis of the unlabeled reaction mixture (Table S1), are indicated. Asterisked products<sup>7</sup> arise late in the reaction, presumably as a result of hydrolysis or transglycosylation with diphospholipid. They do not affect the analysis.

Gal-labeling prevents substrates from reacting at their nonreducing ends these experiments demonstrate that the glycan polymers grow by addition of disaccharide subunits to the reducing end of the growing chain.

Knowing the direction of chain elongation is fundamental to understanding the mechanism of any polymerase. In the case of the PGTs, the direction of glycan chain polymerization is important for thinking about how the bacterial cell wall is built. Accordingly, some of the earliest experiments carried out to understand the process of peptidoglycan synthesis addressed the direction in which the glycan chains elongate.<sup>9</sup> It was suggested that the chains grow from their reducing ends, although the generality of the finding was questioned.<sup>2</sup> Two crystal structures of PGTs have presented models for the polymerization reaction in which the glycan chains were proposed to grow from their reducing ends,<sup>3</sup> but no biochemical support for the models was provided. Since the only experiments addressing directionality involved crude membrane preparations and since similar experiments with other glycan polymerases have been shown to give conflicting, and sometimes erroneous, results,<sup>10</sup> we have assessed the direction of chain elongation using pure PGTs

as well as a new experimental strategy. Our results have established that four different PGTs from a diverse set of Gram-negative and Gram-positive organisms catalyze the addition of Lipid II to the reducing end of the growing peptidoglycan polymer. Given the structural homology of this enzyme family, we propose that the entire class uses a reducing-end mechanism for elongation.

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**Supporting Information Available:** Experimental procedures, supplemental Figures S1, S2, and S3, and Tables S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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