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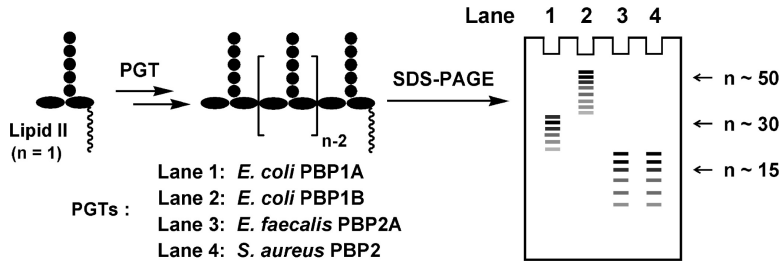
J. Am. Chem. Soc., **2008**, 130 (43), 14068-14069 • DOI: 10.1021/ja806016y • Publication Date (Web): 04 October 2008



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Published on Web 10/04/2008

Isolated Peptidoglycan Glycosyltransferases from Different Organisms Produce Different Glycan Chain Lengths

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Bacterial cells are surrounded by a polymer matrix comprising cross-linked strands of peptidoglycan (PG). This matrix, called the sacculus, functions as an exoskeleton, maintaining cell shape and enabling the plasma membrane to withstand high internal osmotic pressures.[1] The three-dimensional architecture of PG is not yet clear but is presumed to depend, among other things, on the lengths of the glycan strands, which are synthesized by processive enzymes called peptidoglycan glycosyltransferases (PGTs).[2] Numerous studies have evaluated lengths of glycan strands from digested bacterial sacculi, and a range of values have been reported even for digests from the same organism.[3] Because substrates and appropriate analytical methods were not available until recently, there have been no systematic studies comparing the lengths of glycan strands produced by different PGTs *in vitro*. Using a high-resolution gel electrophoresis assay recently developed in our laboratory,[2b] we have performed a comparative study of the glycan strand length distributions produced by four different PGTs, *Escherichia coli* PBP1A (*E. coli* PBP1A), *Escherichia coli* PBP1B (*E. coli* PBP1B), *Enterococcus faecalis* PBP2A (*E. faecalis* PBP2A), and *Staphylococcus aureus* PBP2 (*S. aureus* PBP2) (Figure 1). We show that different PGTs produce glycan chains having a characteristic intrinsic length distribution. The intrinsic lengths are a function of the particular PGT but are independent of enzyme:substrate ratios. There is a correlation between the intrinsic *in vitro* product lengths and the longest strands isolated from sacculi. The implications of these observations for the architecture of the bacterial cell wall are discussed.

The four PGTs we studied were overexpressed, purified, and subjected under similar conditions to reaction with heptaprenyl-[^{14}C]-Lipid II (**1**) (Figure 1a).[2,4−6] Unexpectedly, we found that the four PGTs produced glycan chains of different limiting lengths (i.e., the size beyond which the length does not increase even if reaction times are extended and additional substrate is added) under similar reaction conditions. For *E. coli* PBP1B, the limiting length was ∼50 disaccharide units (Lipid 100), whereas it was ∼30 disaccharide units for *E. coli* PBP1A (Lipid 60) and ∼15 disaccharide units for *E. faecalis* PBP2A and *S. aureus* PBP2 (Lipid 30) (Figure 2). These findings suggest that there are intrinsic differences among the enzymes with respect to the features that control chain length, even though the reaction mechanisms are similar.

To probe the factors that affect polymer length, we evaluated whether enzyme:substrate ratios affect the length distributions. Preliminary experiments showed that the glycan strand length distributions were identical at different enzyme:substrate ratios for each enzyme.[7] The results for *E. faecalis* PBP2A are particularly clear because this enzyme makes relatively short glycan chains (∼15 disaccharide units) that fall within the well-resolved region of the polyacrylamide gel.[4e] We found that there were no significant differences in final product lengths even when the enzyme:substrate ratio varied by a factor of 100 (Figure 3). It might be expected that an enzyme:substrate ratio of 1:1 would yield mainly single-turnover products (e.g., Lipid IV, $n = 2$), but instead, a distribution centered around Lipid 30 (15 disaccharide units) was observed. These results imply that there is a slow step in which a small fraction of the active enzyme in the reaction couples Lipid II subunits to form Lipid IV, and this slow step is followed by a rapid elongation

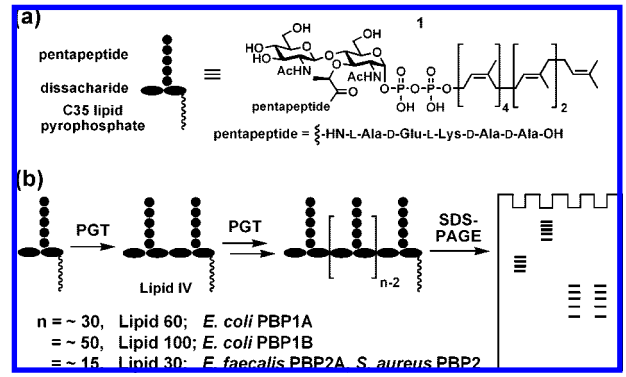

Figure 1. (a) Chemical structure of the heptaprenyl-[^{14}C]-Lipid II analogue **1** used in this study. The ^{14}C radiolabels are incorporated into the GlcNAc residue. (b) Schematic representation of the SDS−PAGE assay.

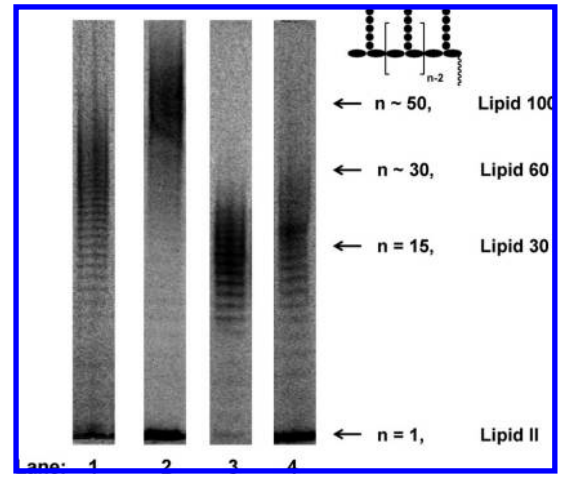

Figure 2. Length distributions of four full-length PGTs: *E. coli* PBP1A (lane 1), *E. coli* PBP1B (lane 2), *E. faecalis* PBP2A (lane 3), and *S. aureus* PBP2 (lane 4). The reactions in lanes 1−3 were quenched after 3 min, and the reaction in lane 4 was quenched after 60 min. The product lengths in each lane were determined by glycan chain ladders for shorter products (data not shown) or retardation factors for longer products, as described in ref 2b.

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10.1021/ja806016y CCC: $40.75

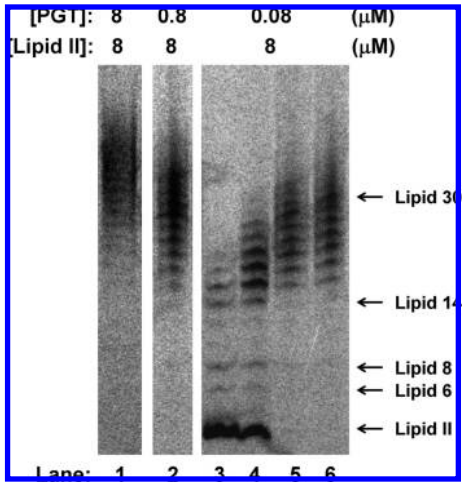

Figure 3. Effect of enzyme:substrate ratio on glycan chain lengths produced by *E. faecalis* Δ TMPBP2A. Concentrations of Lipid II and enzyme used in each reaction are shown. Reactions in lanes 1 and 2 were quenched after 20 min. Reactions in lanes 3−6 were quenched after 3, 5, 20, and 65 min, respectively. The final average lengths at all three enzyme:substrate ratios are around Lipid 30 (15 disaccharide units).

process during which Lipid II subunits are added until the products reach the intrinsic length threshold.[8] A consequence of the slow initiation−fast polymerization process is that PGTs produce long glycan chains even when Lipid II is limiting.

Additional evidence for an intrinsic product length distribution emerges from a comparison of experiments carried out at 1:10 or 1:100 enzyme:substrate ratios. Under these conditions, *E. faecalis* ΔTMPBP2A produced Lipid 30 chains even when the reaction went to complete conversion. If the oligomeric products had not released once the intrinsic length was reached, the observed product length at full conversion for the 1:100 enzyme:substrate ratio would have been ~Lipid 200 (100 disaccharide units), whereas at the 1:10 ratio it would have been Lipid 20 (10 disaccharide units). Taken together, the enzyme:substrate ratio experiments imply that *E. faecalis* ΔTMPBP2A must have some mechanism to facilitate product release at a length of about Lipid 30 so that new oligomers can initiate and polymerize until all the Lipid II is used up. The results obtained for *E. faecalis* ΔTMPBP2A hold for all the other PGTs studied here, which also produce glycan strands of a characteristic length, regardless of enzyme:substrate ratios.

The existence of an intrinsic product length for these processive glycan polymerases, which translocate rather than release products during elongation, implies that there must be a termination/release mechanism that frees the products once the length threshold is achieved. The biological significance of different PGTs producing different length distributions is unclear, but it should be pointed out that most bacteria contain multiple PGTs. Some studies have suggested that the different PGTs act at different stages of cell growth and division,[9] and it is possible that the variation in glycan strand lengths reflects different cellular functions.

In closing, we note that there have been many studies aimed at determining peptidoglycan chain lengths because this information is required to assess models of cell wall architecture.[10,1a] Two extreme models for the structure of the bacterial cell wall have been proposed:[3a,11] in the classical model, the glycan strands are parallel to the bacterial cell membrane; in the more recently proposed scaffold model, they are perpendicular. HPLC profiles of glycan strands isolated from digests of *E. coli* sacculi show *both* long (13 to >31 disaccharide units) and short (2 to 12 disaccharide units) glycan chains.[3b] The short strands may result from sample preparation procedures and/or may reflect processing by lytic enzymes during cell wall synthesis.[3a] Regardless of how these short strands arise, the longer strands isolated from *E. coli* sacculi are similar in length to the intrinsic glycan chain lengths produced by the *E. coli* PGTs *in vitro*. These long strands are only consistent with a model for peptidoglycan architecture in which the glycan chains are parallel to the cell surface. It is possible that the structure of peptidoglycan varies at different locations in the cell, perhaps explaining the presence of both short and long strands.[11d]

Acknowledgment. This work was supported by the NIH (GM076710).

Supporting Information Available: Experimental procedures, including the cloning and purification of *E. faecalis* ΔTMPBP2A and the details of SDS−PAGE assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) Four full-length and one transmembrane (TM) anchor-truncated PGTs (*E. faecalis* ΔTMPBP2A) were used in this study. See the following references for expression and purification conditions. (a) For *E. coli* PBP1A: Zhang, Y.; Fechter, E. J.; Wang, T.-S. A.; Barrett, D.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2007**, *129*, 3080–3081. (b) For *E. coli* PBP1B: Chen, L.; Walker, D.; Sun, B.; Hu, Y.; Walker, S.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5658–5663. (c) For *E. faecalis* PBP2A: Adachi, M.; Zhang, Y.; Leimkuhler, C.; Sun, B.; LaTour, J.; Kahne, D. *J. Am. Chem. Soc.* **2006**, *128*, 14012–14013. (d) For *S. aureus* PBP2: Barrett, D.; Leimkuhler, C.; Chen, L.; Walker, D.; Kahne, D.; Walker, S. *J. Bacteriol.* **2005**, *187*, 2215–2217. (e) See Supporting Information for the cloning and purification of *E. faecalis* ΔTMPBP2A. The TM anchor has no effect on the length distributions of glycan strands produced *in vitro* (see Figure S1). The truncated construct was used for the experiments shown in Figure 3 because it does not aggregate like the full-length construct.
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(6) The amino acid at position three of the Lipid II pentapeptide varies among organisms. In *E. coli.*, the pentapeptide sequence is L-Ala-D-Glu-meso-A_2pm-D-Ala-D-Ala, while in *E. faecalis* and *S. aureus*, it is an L-Lys modified with either L-Ala-L-Ala and $(Gly)_5$, respectively. Previous work has suggested that PGTs are not sensitive to the identity of the amino acid in the third position of the pentapeptide. See the following references. (a) Schwartz, B.; Markwalder, J. A.; Seitz, S. P.; Wang, Y.; Stein, R. L. *Biochemistry* **2002**, *41*, 12552–12561. (b) Liu, H.; Wong, C.-H. *Bioorg. Med. Chem.* **2006**, *14*, 7187–7195.
(7) To verify the accuracy of the enzyme:substrate ratio, the enzyme concentration was determined by active-site titration with moenomycin, as described in ref. 2b. *E. coli* PBP1A and *E. faecalis* ΔTMPBP2A were >90% active.
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JA806016Y