The role of the substrate lipid in processive glycan polymerization by the peptidoglycan glycosyltransferases

Deborah L. Perlstein,[†] Tsung-Shing A. Wang, [‡] Emma H. Doud, ,[†‡] Daniel Kahne*,[‡] and Suzanne Walker*,[†].

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

and

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

kahne@chemistry.harvard.edu; suzanne_walker@hms.harvard.edu

Supporting information

Materials: Lipid II (**1b-g**), Lipid IV (**2b**), and Gal-LP4 (**3b**) were synthesized as described previously.[1,2] *E. coli* PBP1A (amino acids 26-850 with C-terminal His-tag), *E. coli* PBP1B, and *A. aeolicus* PBP1A (AaPGT; thioredoxin fusion to the PGT domain consisting of amino acids 29 -243), were purified as described previously.[2,3,4] Moenomycin A (MmA) was isolated from flavomycin feedstock as described.[5] All other chemicals were obtained from Sigma-Aldrich.

Acceptor site assays (Figure 3A): For the short lipid analogs **1d**, **1f**, and **1g**, reactions contained 1.5 μM AaPGT, 5 μM [^{14}C]-GLP4 (**3b,** [^{14}C]-galactose) and 3 μM [^{14}C]-LP2 ([^{14}C]-GlcNAc) analog in 50 mM HEPES (pH 7.5), 10 mM $CaCl_2$, 20% DMSO (assay buffer). Reactions were initiated by the addition of enzyme following pre-incubation at 55˚C. Reactions were quenched by the addition of excess MmA and analyzed by SDS-PAGE as described previously except 12% mini-gels (8.3 x 6.8 cm; 0.75 mm thickness) were used instead of 9% (20 x 20 cm; 1 mm) gels.[3] For the gels shown in Figure 3A, reactions were quenched at 5 min, 10 min, and 30 min for **1d**, **1f**, and **1g**, respectively.

For LP2 analogs **1e** and **1c**, AaPGT (1 μM) was incubated with [^{14}C]-LP2 (8 μM **1e** or **1c**) and [^{14}C]-**3b** (10 μM) in the PGT assay buffer. Reactions were incubated at 55˚C and quenched by the addition of excess MmA. Product formation was monitored via the SDS-PAGE assay on 20 x 20 cm; 9% gels as described previously.[3] Reactions shown in Figure 3A were quenched at 25 min and 12 min for **1e** and **1c**, respectively.

Short lipid analogs are acceptors with other PGTs. Although most of the acceptor assays were completed with AaPGT, experiments with two additional PGTs from *E. coli* (PBP1A and

PBP1B) demonstrated that the observed acceptor substrate flexibility was not exclusive to the AaPGT domain.

***1f** is an acceptor substrate for E. coli PBP1B: E. coli* PBP1B[6] (0.15 μM) was incubated with **1f** (4 μM) and **3b** (4 μM) in an assay buffer containing 50 mM HEPES at pH 7.5, 10 mM $CaCl_2$, 1 kU/mL penicillin G, 0.2 mM decyl PEG and 11% DMSO. Reactions were incubated for 40 min at room temperature, quenched with MmA and analyzed by SDS-PAGE mini gels as described above (Figure S1A).

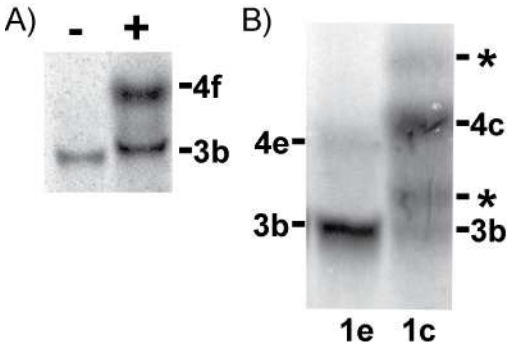

***Figure S1.** Acceptor site assays with E. coli PBP1B (A) and PBP1A (B). A)* Radiolabeled GLP4 (**3b**) and citronellyl-LP2 (**1f**) were incubated in the presence (+) and absence (-) of *E. coli* PBP1B. Only one new product was formed with migratory properties of GLP6 (**4f**). *B)* Radiolabeled GLP4 (**3b**) was incubated with either **1e** or **1c** in the presence of *E. coli* PBP1A. In both cases, one major product was formed (GLP6, **4e** or **4c** for reactions with **1e** and **1c**, respectively). Reactions with **3b** and **1c** produced at least two additional radiolabeled products (*).

***1e** and **1c** are acceptor substrates for E. coli PBP1A:* PBP1A (0.5 μM) was incubated with GLP4 (**3b**, 8 μM) and the LP2 analog (8 μM) in the PGT assay buffer supplemented with 1 kU/mL penicillin G. Following 10 min incubation at room temperature, reactions were quenched by the addition of excess MmA and analyzed on 20 x 20 cm gels as described previously.[3] We noted that in reactions with **1c**, two additional radiolabeled products were

observed (*, Figure S1B). The formation of additional minor products with similar migratory properties were also observed in reactions with AaPBP1A (data not shown). Based on our high resolution mass spectrometry analysis of these reactions (see below), we propose these additional bands are C20-LP4 (**2c**) and C20-GLP8, for the faster and slower migrating products, respectively.

*Changing the ratio of GLP4 (**3b**):LP2(**1f**) does not produce any products longer than GLP6.* AaPGT (6 μM) was incubated with [^{14}C]-GLP4 (**3b**, 2 μM) and cold citronellyl-LP2 (**1f** at 2.5, 5, or 10 μM) in the assay buffer. Following incubation for 30 min at 55˚C, reaction was quenched and analyzed via SDS-PAGE. The results are shown in Figure S2. For comparison, a reaction of **3b** (4 μM) with **1b** (16 μM) with *E. coli* PBP1B (0.8 μM) is shown (gel taken from supplementary material of Ref. 2). Clearly, no additional higher molecular weight products are seen in reactions of with **1f** whereas numerous elongated products are seen with **1b**.

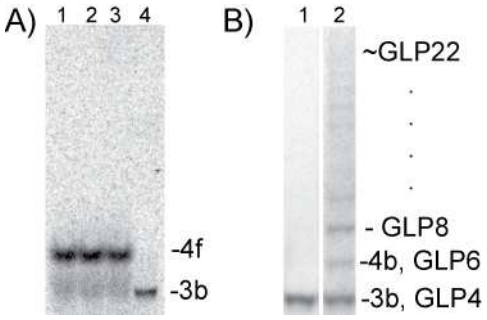

Figure S2. Short lipid analog **1f** cannot undergo multiple rounds of coupling. *A)* SDS-PAGE analysis of reactions of [^{14}C]-**3b** with **1f**. For lanes 1, 2, and 3, the molar ratio of **3b**:**1f** is 1:1.25, 1:2.5 and 1:5, respectively. Lane 4 has just **3b** for reference. *B)* SDS-PAGE analysis of reactions of of [^{14}C]-**3b** with **1b** (Lane 2). Lane 1 is **3b**, shown for reference.

Homopolymerization assays (Figure 3B). Reactions (5 μL) contained AaPGT and [^{14}C]-LP2 in assay buffer. Concentrations of assay components and time of incubation are listed in Table S1.

Following incubation at 55˚C, the reaction was quenched by addition of MmA. Products were analyzed via the SDS-PAGE assay on a 20 x 20 cm gel.[3]

Table S1. Conditions for homopolymerization reactions in Figure 3B.

LP2	**[LP2], μM**	**[AaPGT], μM**	**Time, min**
C10-LP2 (**1e**)	8 μM	10 μM	60 min
C20-LP2 (**1c**)	10 μM	1 μM	60 min
C35-LP2 (**1b**)	8 μM	0.08 μM	10 min

*Mass Spec analysis demonstrating substrates with a C20 lipid (**c**) can react from both the donor and acceptor site.* Truncated *E. coli* PBP1A (0.5 μM) was incubated with **1c** (8 μM) and gal-labeled substrate (8 μM, **3b** or heptaprenyl(**b**)-GLP2) in PGT assay buffer supplemented with penicillin G (1 kU/mL). Reactions (15 μL) were incubated for 30 min at room temperature. Enzymatic assays were quenched by boiling and centrifuged to remove precipitated protein. Reactions were analyzed via high resolution mass spectrometry (6520 Accurate Mass Q-ToF LC/MS, Agilent). Samples were separated on a C18 column (50x2mm; Gemini-NX 5μ 110A; Phenomenex) eluted with 100%A (95:5 Water:Methanol/0.1% NH_4OH) for 2.5 min followed by a linear gradient to a 100%B (60:35:5 Isopropanol:Methanol:Water/0.1% NH_4OH) over 9.5 min at a flow rate of 0.4 mL/min. Mass spectra were analyzed with Agilent's MassHunter software package. PGT substrates and products were identified by exact mass. Expected and observed *m/z* are listed in Table S2 and Table S3. The most abundant ion for each proposed product is shown in Figure S3.

Table S2. Reactions of GLP4 (**3b**) and C20-LP2 (**1c**).

Reaction time	Compound	Retention time (min)	ion	Observed (calculated)
0.5 min	GLP4	8.493	$[M-2H]^{-2}$ $[M-3H]^{-3}$	1355.6379 (1355.6386) 903.4223 (903.4233)
	C20LP2	7.175	$[M-H]^{-1}$ $[M-2H]^{-2}$	1397.6147(1397.6151) 698.3046 (698.3039)
30 min	GLP4	8.505	$[M-2H]^{-2}$ $[M-3H]^{-3}$	1355.6387 (1355.6386) 903.4220 (903.4233)
	C20LP2	7.122	$[M-2H]^{-2}$	698.3046 (698.3039)
	C20-GLP6	6.086	$[M-2H]^{-2}$ $[M-3H]^{-3}$ $[M-4H]^{-4}$ $[M-5H]^{-5}$	1727.7524 (1727.7591) 1151.5025 (1151.5036) 863.3753 (863.3759) 690.5002 (690.4993)
	C20-GLP8	5.969	$[M-3H]^{-3}$ $[M-4H]^{-4}$ $[M-6H]^{-6}$	1467.6398 (1467.6466) 1100.4833 (1100.4831) 733.3196 (733.3196)

Table S3. Reactions of GLP2 and C20-LP2 (**1c**)

Reaction time	Compound	Retention time (min)	ion	Observed (calculated)
0.5 min	GLP2	9.229	$[M-2H]^{-2}$ $[M-3H]^{-3}$	881.4242 (881.4242) 587.2811 (587.2804)
	C20LP2	7.111	$[M-H]^{-1}$ $[M-2H]^{-2}$	1397.6123(1397.6151) 698.3052 (698.3039)
30 min	GLP2	9.212	$[M-2H]^{-2}$ $[M-3H]^{-3}$	881.4249 (881.4242) 587.2810 (587.2804)
	C20LP2	7.122	$[M-H]^{-1}$ $[M-2H]^{-2}$	1397.6126(1397.6151) 698.3052 (698.3039)
	C20-GLP4	6.343	$[M-3H]^{-3}$ $[M-4H]^{-4}$	835.3609 (835.3607) 626.2696 (626.2687)
	C20-GLP6	6.026	$[M-3H]^{-3}$ $[M-4H]^{-4}$ $[M-5H]^{-5}$	1151.5030 (1151.5036) 863.3776 (863.3759) 690.5007 (690.4993)
	C20-GLP8	5.909	$[M-3H]^{-3}$ $[M-4H]^{-4}$ $[M-6H]^{-6}$	1467.6498 (1467.6466) 1100.4835 (1100.4831) 733.3203 (733.3196)

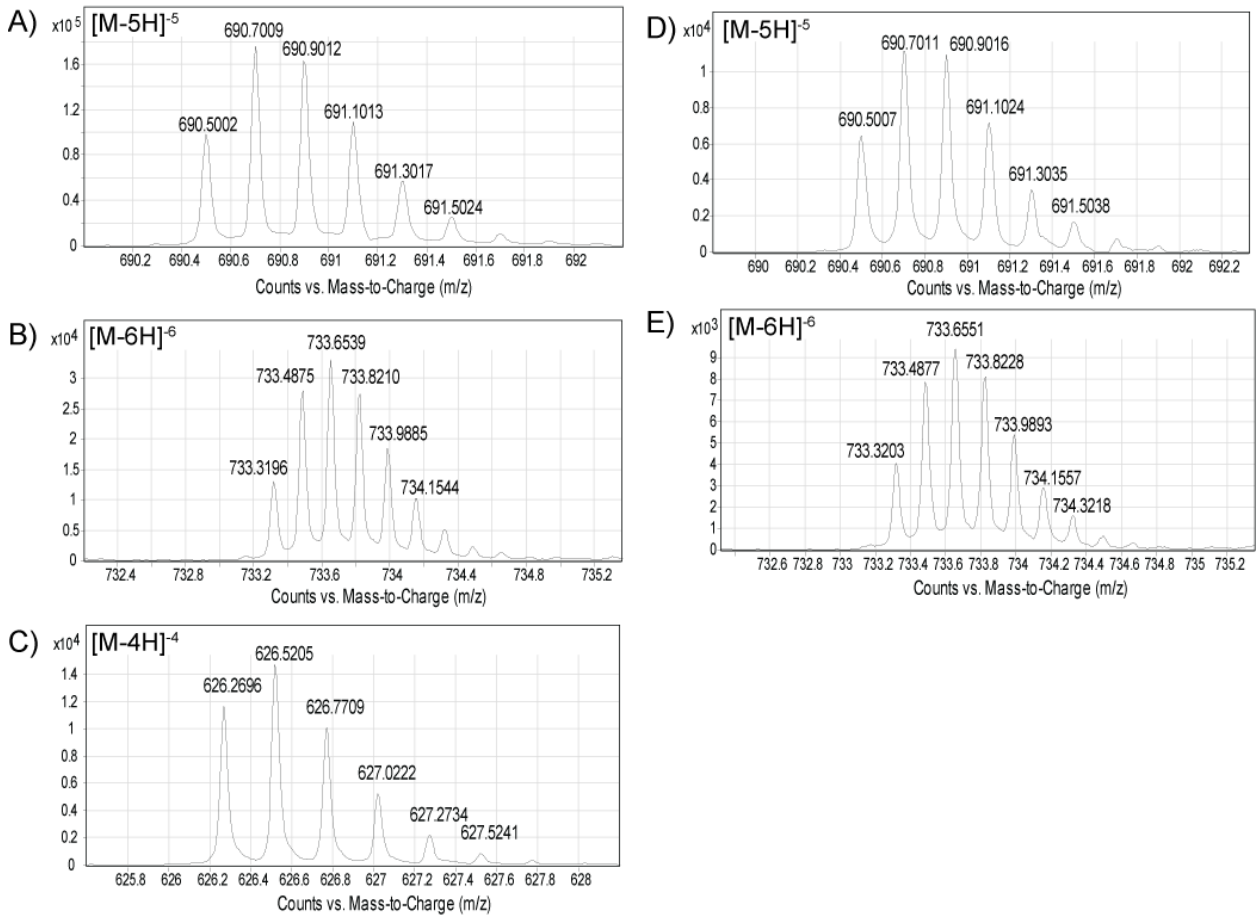

Figure S3. Mass spectra of C20-lipid coupling products. For reactions with GLP4 (**3b**) and C20-LP2 (**1c**), only two products were observed, C20-LP6 (*A*) and C20-GLP8 (*B*). For reactions with heptaprenyl(**b**)-GLP2 and C20-LP2 (**1c**), the following products are observed: C20-GLP4 (*C*), C20-GLP6 (*D*), and C20-GLP8 (*E*). For each product, only the most abundant ion is shown.

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