

# Reconstitution of Peptidoglycan Cross-Linking Leads to Improved Fluorescent Probes of Cell Wall Synthesis

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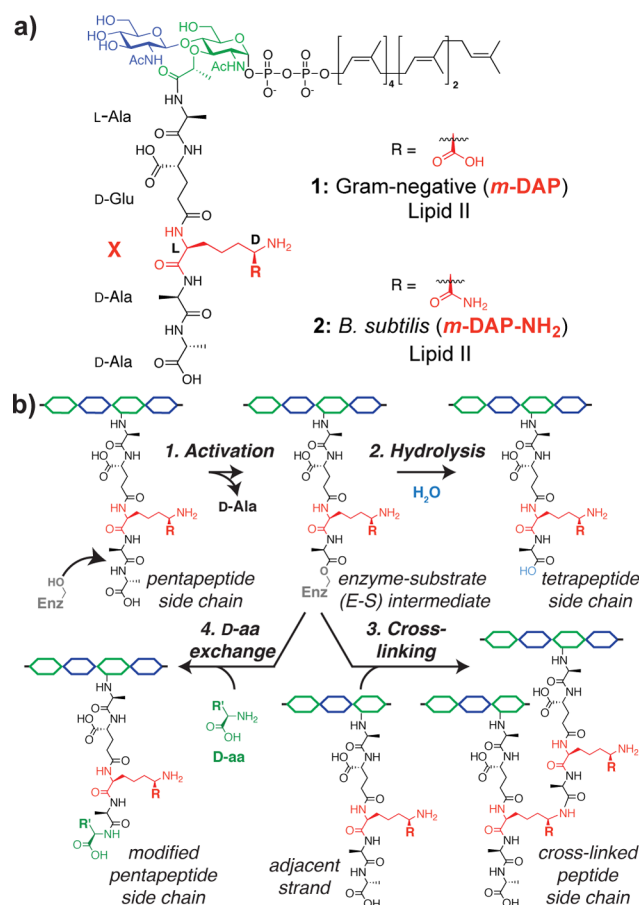
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## Supporting Information

**ABSTRACT:** The peptidoglycan precursor, Lipid II, produced in the model Gram-positive bacterium *Bacillus subtilis* differs from Lipid II found in Gram-negative bacteria such as *Escherichia coli* by a single amidation on the peptide side chain. How this difference affects the cross-linking activity of penicillin-binding proteins (PBPs) that assemble peptidoglycan in cells has not been investigated because *B. subtilis* Lipid II was not previously available. Here we report the synthesis of *B. subtilis* Lipid II and its use by purified *B. subtilis* PBP1 and *E. coli* PBP1A. While enzymes from both organisms assembled *B. subtilis* Lipid II into glycan strands, only the *B. subtilis* enzyme cross-linked the strands. Furthermore, *B. subtilis* PBP1 catalyzed the exchange of both D-amino acids and D-amino carboxamides into nascent peptidoglycan, but the *E. coli* enzyme only exchanged D-amino acids. We exploited these observations to design a fluorescent D-amino carboxamide probe to label *B. subtilis* PG in vivo and found that this probe labels the cell wall dramatically better than existing reagents.

Bacterial cells are surrounded by a cell wall composed of layers of peptidoglycan (PG). This mesh-like macromolecule stabilizes cell membranes against high internal osmotic pressures and is essential for survival. Peptidoglycan is assembled from the lipid-linked disaccharide pentapeptide precursor Lipid II (Figure 1a, 1).<sup>1</sup> Peptidoglycan glycosyltransferases polymerize Lipid II into glycan strands and enzymes known as penicillin-binding proteins (PBPs) cross-link and process the peptide side chains to produce mature PG.<sup>2</sup> Because PG is highly conserved and essential for cell survival, it is a target for antibiotics. Indeed, the most successful class of antibiotics in history, the  $\beta$ -lactams, inhibits the transpeptidases (TPs) that cross-link the carbohydrate strands of PG.<sup>3</sup>

TPs contain a conserved active-site serine that attacks the terminal D-Ala-D-Ala amide bond of the pentapeptide attached to the glycan polymer, forming a covalent enzyme–substrate (E-S) intermediate and releasing D-Ala (Figure 1b, activation step). An amino group on the peptide side chain of another glycan strand can then attack the E-S intermediate to form a cross-link and regenerate the enzyme (Figure 1b, cross-linking



**Figure 1.** Reactions catalyzed by transpeptidases (TPs) that cross-link peptidoglycan. (a) Structures of Lipid II used by *E. coli* and *B. subtilis*.<sup>4</sup> (b) Reactions catalyzed by TPs proceed via an enzyme–substrate intermediate that can be resolved by water to generate a tetrapeptide side chain, by a side chain on another glycan strand to yield a cross-link, or by a D-amino acid to give a pentapeptide with a new terminal amino acid.

step). While the structure of Lipid II is highly conserved, the identity of the amino acid containing the reactive amino group

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varies by species.<sup>1a,5</sup> As the Lipid II substrate is difficult to obtain,<sup>6,7</sup> it is not well understood how these differences in the stem peptide affect TP cross-linking chemistry.<sup>8,9</sup>

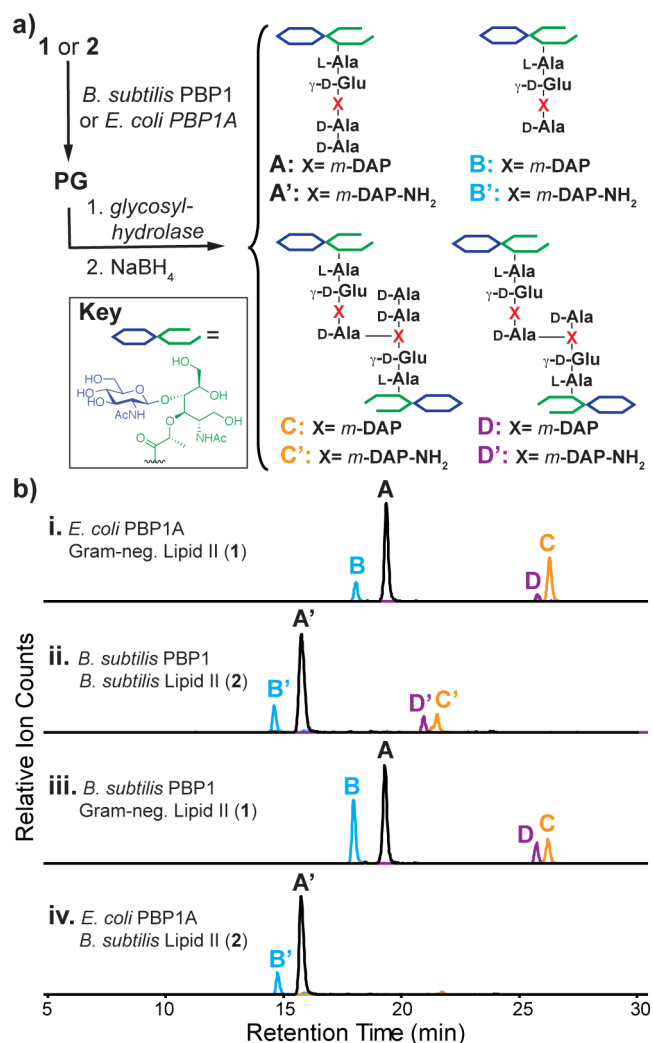
The canonical Lipid II used by Gram-negative bacteria, including *Escherichia coli*, contains the pentapeptide L-Ala- $\gamma$ -D-Glu-*m*-DAP-D-Ala-D-Ala (1).<sup>10</sup> The third residue, *m*-DAP, which functions as the nucleophile in the cross-linking reaction, has a D-stereocenter in the side chain. *Bacillus subtilis* Lipid II is similar but contains a carboxamide instead of a carboxylic acid on the *m*-DAP side chain (2).<sup>11,12</sup> Cell wall precursor 1 has been synthesized previously.<sup>8d</sup> We synthesized the *B. subtilis* Lipid II substrate 2 in 32 steps by adapting the synthesis of Gram-negative Lipid II<sup>8d,13</sup> to introduce the carboxamide-containing *m*-DAP residue.<sup>14</sup>

Access to both 1 and 2 has allowed us to investigate the substrate preferences of TPs from *E. coli* and *B. subtilis*. *E. coli* PBP1A and *B. subtilis* PBP1<sup>15</sup> were purified as described in the Supporting Information, and each enzyme was then incubated with substrate 1 or substrate 2. These enzymes contain an N-terminal glycosyltransferase domain that polymerizes disaccharide subunits and a C-terminal TP domain that cross-links polymeric glycan strands. To assess cross-linking, we treated reaction mixtures with the glycosylhydrolase mutanolysin followed by NaBH<sub>4</sub> and then used LC/MS to identify the products (Figure 2a).<sup>8d,16</sup> As expected, each enzyme is able to polymerize and cross-link its native substrate (Figure 2b, traces i and ii). In addition, *B. subtilis* PBP1 is able to cross-link the canonical Gram-negative Lipid II (1, Figure 2b, trace iii), but *E. coli* PBP1A is not able to cross-link *B. subtilis* Lipid II (2, Figure 2b, trace iv), implying that the *E. coli* PBP discriminates against the carboxamide on the *m*-DAP.

We have previously shown that *E. coli* PBP1A can exchange the terminal D-Ala in cell wall precursors with other D-amino acids during in vitro PG synthesis.<sup>13a,16,17</sup> To further assess the substrate scope of the *E. coli* and *B. subtilis* enzymes, we examined their ability to incorporate either D-Phe or D-Phe carboxamide (D-Phe-NH<sub>2</sub>) into synthetic PG (Figure 3a). *E. coli* PBP1A incorporated D-Phe but not D-Phe-NH<sub>2</sub> (Figure 3b, traces i and ii), whereas *B. subtilis* PBP1 incorporated both (Figure 3b, traces iii and iv).<sup>18</sup> Hence, the cross-linking and D-amino acid incorporation experiments are consistent in showing that the *E. coli* PBP1A TP domain discriminates against carboxamide substrates but the *B. subtilis* PBP1 TP domain does not.

A variety of D-amino acid probes have recently been developed to fluorescently label PG in living cells,<sup>19</sup> but labeling is poor in *B. subtilis* unless the cell wall hydrolase DacA, which removes terminal D-amino acids from PG, is absent.<sup>19a</sup> To determine if D-amino carboxamides can be stably incorporated into PG during *B. subtilis* growth, we grew cells to early log phase in medium supplemented with 500  $\mu$ M D-Phe or D-Phe-NH<sub>2</sub> and then analyzed the composition of the pentapeptide in PG fragments following enzymatic degradation. LC/MS analysis showed that D-Phe and D-Phe-NH<sub>2</sub> were incorporated exclusively at the fifth position of the stem peptide, as observed previously for D-amino acids,<sup>20</sup> but the levels of D-Phe-NH<sub>2</sub> were much higher (Figure S1). These results led us to examine the utility of fluorescent D-amino carboxamide probes for imaging cell wall biosynthesis in *B. subtilis*.

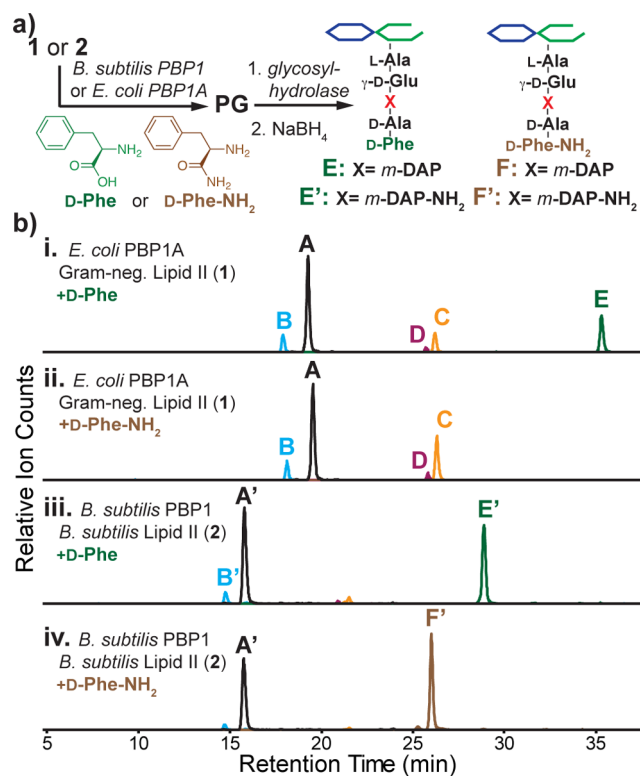
We prepared previously reported fluorescent probe 3 (FDL)<sup>19a</sup> as well as new probe 4 (FDL-NH<sub>2</sub>) by appending fluorescein to D-Lys and D-Lys carboxamide, respectively



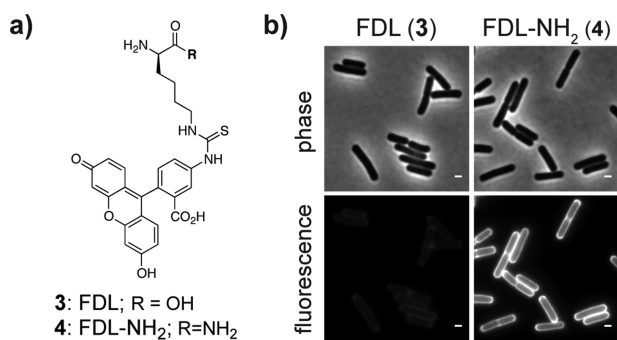
**Figure 2.** *B. subtilis* transpeptidases (TPs) can cross-link amidated peptidoglycan precursors in vitro. (a) Protocol for detecting cross-linking by *E. coli* and *B. subtilis* TPs in vitro. Fragments A/A' are derived from unmodified polymer; the remaining products are derived from polymer that has been acted on by a TP. (b) LC/MS-extracted ion chromatograms showing unprocessed (A/A'), hydrolyzed (B/B'), and cross-linked products (C/C', D/D') formed in the presence of *E. coli* PBP1A (i,iv) or *B. subtilis* PBP1 (ii,iii) and substrate 1 (i,iii) or 2 (ii,iv).

(Figure 4a). Wild-type *B. subtilis* (dacA+) was grown in medium supplemented with 100  $\mu$ M of either 3 or 4 for approximately four generations, and cells were then examined by fluorescence microscopy. FDL-NH<sub>2</sub> 4 efficiently labeled both new septa and the cylindrical walls of the cells. By contrast, FDL 3 inefficiently labeled the cells (Figure 4b). Even FDL-NH<sub>2</sub> concentrations as low as 10  $\mu$ M yielded strong labeling (Figure S2). Use of the fluorescent carboxamide probe FDL-NH<sub>2</sub> may be preferable for imaging cell wall synthesis, as it does not require mutations (e.g., *dacA* deletion) that perturb peptidoglycan processing.

To test whether D-amino carboxamides label PG in *E. coli*, we grew cells to early log phase in medium with 500  $\mu$ M of either D-Phe or D-Phe-NH<sub>2</sub>, harvested the PG, and analyzed the PG fragments using LC/MS as before. As reported previously for D-amino acids,<sup>20b,21</sup> D-Phe incorporation was detected exclusively in the fourth position of the stem peptide (Figure S3b, trace ii). Strikingly, almost no D-Phe-NH<sub>2</sub> incorporation was observed



**Figure 3.** *B. subtilis* TPs can incorporate D-amino carboxamides into PG in vitro. (a) Protocol for detecting TP-mediated D-amino acid or carboxamide exchange in vitro. (b) LC/MS-extracted ion chromatograms of products generated by reaction of *E. coli* PBP1A (i,ii) or *B. subtilis* PBP1 (iii,iv) with Lipid II and 1 mM D-Phe (i,iii) or D-Phe-NH<sub>2</sub> (ii,iv). Products E/E' contain D-Phe; product F' contains D-Phe-NH<sub>2</sub>.



**Figure 4.** Fluorescent D-amino carboxamide probes dramatically improve detection of PG synthesis. (a) Structures of fluorescent probes. (b) *B. subtilis* was grown with 100 μM probe to mid log phase. Images were adjusted to the same intensity scale to allow comparison. Scale bars: 1 μm.

(Figure S3b, trace iii). It has previously been suggested that another subset of TPs called the L<sub>D</sub>-transpeptidases are responsible for fourth-position incorporation,<sup>20b</sup> and these data suggest that, like the *E. coli* PBPs, *E. coli* L<sub>D</sub>-transpeptidases discriminate against carboxamides. Likewise, *E. coli* grown with 500 μM of either probe 3 or 4 for four generations incorporated 3 but not 4 (Figure S4), indicating that the ability to incorporate D-amino carboxamides may be specific to organisms like *B. subtilis* that cross-link substrates containing an amino group alpha to a carboxamide.

The ability of enzymes from *B. subtilis* to incorporate both D-amino acids and D-amino carboxamides suggests that there may

be two trajectories for nucleophiles to interact with the *B. subtilis* transpeptidase enzyme–substrate intermediate: one for D-amino acids, which may constitute the reverse of the activation step in which a D-amino acid is the leaving group, and the other for forming cross-links to incoming *m*-DAP-NH<sub>2</sub> side chains as well as D-amino carboxamides that resemble these side chains. For *E. coli*, the incoming side chain is essentially a D-amino acid, and other modifications are not well tolerated. The dramatically increased efficiency of incorporation of D-amino carboxamides in *B. subtilis* cells could be due to more efficient incorporation of this nucleophile, less efficient removal by endogenous PG hydrolases once installed, or both. D-amino carboxamide probes may also be useful for studying pathogenic bacteria that contain an amidated *m*-DAP residue in their peptidoglycan, such as *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*.<sup>19b,22</sup> Using this general strategy, it may be possible to design probes that mimic cross-linking residues for other bacteria.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Experimental procedures, synthesis of substrates and compound analysis, protein purification protocols, and LC/MS analysis of PG polymers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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