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

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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). 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. 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 β-lactams, inhibits the transpeptidases (TPs) that cross-link the carbohydrate strands of PG.

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 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. As the Lipid II substrate is difficult to obtain, it is not well understood how these differences in the stem peptide affect TP cross-linking chemistry.

The canonical Lipid II used by Gram-negative bacteria, including Escherichia coli, contains the pentapeptide L-Ala-γ-D-Glu-m-DAP-D-Ala-D-Ala (1). 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). Cell wall precursor 1 has been synthesized previously. We synthesized the Bacillus subtilis Lipid II substrate 2 in 32 steps by adapting the synthesis of Gram-negative Lipid II to introduce the carboxamide-containing m-DAP residue.

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 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 glycosylhydrolyase mutanolysin followed by NaBH₄ and then used LC/MS to identify the products (Figure 2a). 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 Bacillus 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. 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-NH₂) into synthetic PG (Figure 3a). E. coli PBP1A incorporated d-Phe but not d-Phe-NH₂ (Figure 3b, traces i and ii), whereas B. subtilis PBP1 incorporated both (Figure 3b, traces iii and iv). 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, but labeling is poor in B. subtilis unless the cell wall hydrolase DacA, which removes terminal d-amino acids from PG, is absent. 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 μM d-Phe or d-Phe-NH₂ and then analyzed the composition of the pentapeptide in PG fragments following enzymatic degradation. LC/MS analysis showed that d-Phe and d-Phe-NH₂ were incorporated exclusively at the fifth position of the stem peptide, as observed previously for d-amino acids, but the levels of d-Phe-NH₂ 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) as well as new probe 4 (FDL-NH₂) by appending fluorescein to d-Lys and d-Lys carboxamide, respectively (Figure 4a). Wild-type B. subtilis (dacA+) was grown in medium supplemented with 100 μM of either 3 or 4 for approximately four generations, and cells were then examined by fluorescence microscopy. FDL-NH₂ efficiently labeled both new septa and the cylindrical walls of the cells. By contrast, FDL 3 inefficiently labeled the cells (Figure 4b). Even FDL-NH₂ concentrations as low as 10 μM yielded strong labeling (Figure S2). Use of the fluorescent carboxamide probe FDL-NH₂ 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 μM of either d-Phe or d-Phe-NH₂ harvested the PG, and analyzed the PG fragments using LC/MS as before. As reported previously for d-amino acids, d-Phe incorporation was detected exclusively in the fourth position of the stem peptide (Figure S3b, trace ii). Strikingly, almost no d-Phe-NH₂ incorporation was observed.
Communication

1. Incorporation of D-amino carboxamides by PBPs

2. Fluorescent D-amino carboxamide probes

3. Supporting Information

4. Author Information

5. Notes

6. Acknowledgments

7. References

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4. A heptaprenyl lipid chain was used rather than the most commonly found natural lipid (undecaprenyl) because the former does not aggregate as extensively and thus is a better substrate for biochemical studies.
