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An Activity-Based Probe for Studying Crosslinking in Live Bacteria**

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Abstract: *Penicillin-binding proteins (PBPs) catalyze the crosslinking of peptidoglycan (PG), an essential process for bacterial growth and survival, and a common antibiotic target. Yet, despite its importance, little is known about the spatiotemporal aspects of crosslinking—largely because of a lack of experimental tools for studying the reaction in live bacteria. Here we introduce such a tool: an activity-based probe that enables visualization and relative quantitation of crosslinking in vivo. In Staphylococcus aureus, we show that fluorescent mimics of the natural substrate of PBPs (PG stem peptide) are covalently incorporated into the cell wall, installing fluorophores in place of natural crosslinks. These fluorescent stem peptide mimics (FSPMs) are selectively recognized by a single PBP in S. aureus: PBP4. Thus, we were able to use FSPM pulse-labeling to localize PBP4 activity in live cells, showing that it is recruited to the septum in a manner dependent on wall teichoic acid.*

The peptidoglycan (PG) cell wall is a meshlike macromolecule that surrounds bacterial cells, providing structure and physical protection. Penicillin-binding proteins (PBPs) mediate the biogenesis of cell walls through two enzymatic activities: transglycosylation and transpeptidation.[1] The former involves incorporation of lipid II monomers into growing PG strands, while the latter gives rise to peptide crosslinks between adjacent strands, a process that confers tensile strength to the PG sacculus and resistance to osmotic stress (Figure S1 in the Supporting Information).

The transpeptidation reaction (crosslinking) occurs in two fundamental steps. First, a PBP cleaves the terminal D-Ala from a donor strand (the stem peptide) and forms an acyl-enzyme intermediate. Next, the PBP ligates an acceptor strand (e.g., the bridge peptide in *Staphylococcus aureus*) to the donor strand, thus leading to the enzyme's regeneration. The resulting crosslinks are essential for bacterial growth and survival, and as such represent a prime target for antibiotic therapy. Indeed, drugs that inhibit PBP transpeptidase activity, such as β-lactams and vancomycin, are the most commonly used antibiotics in the clinic today.[2]

Yet, despite the fundamental importance of crosslinking in both bacterial cell biology and clinical medicine, little is known about the spatiotemporal aspects of this process within the cell. A major reason for this knowledge gap is the lack of experimental tools for interrogating PBP activity in intact bacteria. Fluorescent derivatives of β-lactam antibiotics, which bind to the active site of PBPs,[3] have previously been used to study crosslinking, but such antibiotic-based probes are inherently disruptive to the cell wall, even over the span of a few minutes, and are therefore problematic for live cell analysis.[4] Transpeptidation may also be studied using a biochemical assay in which cell walls are enzymatically digested to produce a dispersion of variably crosslinked muropeptides, which are then quantified by high-performance liquid chromatography (HPLC) to give an overall measure of crosslinking in a bacterial population.[5] However, this "postlysis" experimental approach clearly precludes analysis of the subcellular localization and kinetics of transpeptidation.

In 2012, Kuru et al. introduced a powerful new class of probes called fluorescent D-amino acids (FDAAs), which are covalently incorporated into the PG stem peptide in a wide range of bacterial species (Figure S1).[6] These compounds offer a facile and nontoxic means of "metabolically labeling" sites of new cell wall synthesis.[7] The mechanism by which

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FDAAs are ligated to PG involves an exchange reaction mediated by transpeptidases, wherein FDAAs serve as a surrogate acceptor strand in the transpeptidation reaction (Figure S1).[6] Thus, physiologically, FDAA labeling appears to represent D-amino acid exchange at the distal stem peptide rather than ligation of adjacent PG strands.[8] Therefore, while highly useful for examining nascent PG synthesis, this labeling method may not be the optimal approach for studying crosslinking per se.

In order to interrogate crosslinking more directly, we set out to develop a chemical probe that would serve as a donor strand in the transpeptidation reaction. We hypothesized that a fluorescently tagged mimic of the endogenous substrate of PBPs—the PG stem peptide—would be recognized by transpeptidases and covalently attached to the bridge peptide in place of a natural crosslink (Figure S1). Labeling with these fluorescent stem peptide mimics (FSPMs) would therefore lead to installation of fluorophores precisely at sites of crosslinking (Figure S1), providing a means of studying this crucial enzymatic process in vivo.

To test our hypothesis, we used *Staphylococcus aureus*, an important human pathogen and a well-studied model organism for PG synthesis in gram-positive cocci. *S. aureus* expresses a limited array of PBPs: PBP1–4 in methicillin-sensitive strains.[9] PBP1 is a class B enzyme that possesses two domains, both of which are essential for *S. aureus* viability: an N-terminal scaffolding domain necessary for the initiation of septation, and a C-terminal transpeptidase.[10] PBP2 is a class A enzyme that possesses transpeptidase and transglycosylase activity, both of which are essential for cell growth and survival.[11] PBP3 is a nonessential class B PBP with a single transpeptidase domain that appears to contribute marginally to PG synthesis.[12] PBP4 (class C) is also a nonessential transpeptidase, but unlike PBP3, it plays a major role in crosslinking the staphylococcal cell wall. PBP4 is recruited to the division septum by metabolites of wall teichoic acid (WTA), an anionic cell surface glycopolymer.[13] There, PBP4 works in concert with PBP2 to generate the high degree of crosslinking characteristic of *S. aureus* PG,[14] and also contributes importantly to β-lactam antibiotic resistance.[15] In addition to these four PBPs, methicillin-resistant *S. aureus* (MRSA) strains have acquired a fifth isoform, PBP2a (class B), which has a low affinity for β-lactams and therefore allows continued transpeptidation in the presence of these drugs.[9,16]

Based on the sequence of the *S. aureus* stem peptide (Figure S1), we designed our probe to include the PBP recognition motif, (L-Lys)–(D-Ala)–(D-Ala), which is linked to a fluorophore by a short, flexible peptide, (L-Lys)–(Gly)–(Gly), to furnish the functional FSPM (Figure 1 a). We opted for this minimal tripeptide motif rather than a longer mimic of the stem peptide both for synthetic ease and based on several previous reports—including X-ray crystallographic and in vitro enzymological studies—showing that tripeptide substrates are sufficient for recognition by PBPs.[17]

In proof-of-principle experiments, we used flow cytometry to demonstrate that the fluorescein isothiocyanate (FITC) conjugate of this probe, D-FL, is robustly incorporated by growing *S. aureus* (Figure S2). A diastereomeric control probe L-FL (Figure 1 a) produced only low-level fluorescence, thus indicating that probe labeling is due to stereoselective incorporation rather than nonspecific sticking, consistent with an enzymatic process (Figure S2). Time-course and concentration-dependence studies were then performed to establish standard labeling conditions (Figures S3–S5). Importantly, we found that D-FL produces minimal physiologic perturbation under these labeling conditions, both in terms of growth kinetics and muropeptide composition (Figures S6 and S7).

Next, we labeled *S. aureus* with the more photostable D-A568 conjugate (Figure 1 a) and performed imaging experiments using structured illumination microcopy (SIM) to show that FSPMs localize to the cell wall (Figure 1 b). As a marker for the cell wall, we used Van-A488 (see the Supporting Information for the structure), a fluorescent derivative of vancomycin that binds to the (D-Ala)–(D-Ala) motif within intact PG stem peptides, which are present throughout the cell wall in *S. aureus*.[18] Notably, the diastereomeric control L-A568 (Figure 1 a) produced only minimal labeling in these experiments, further confirming the stereoselectivity of FSPM incorporation.

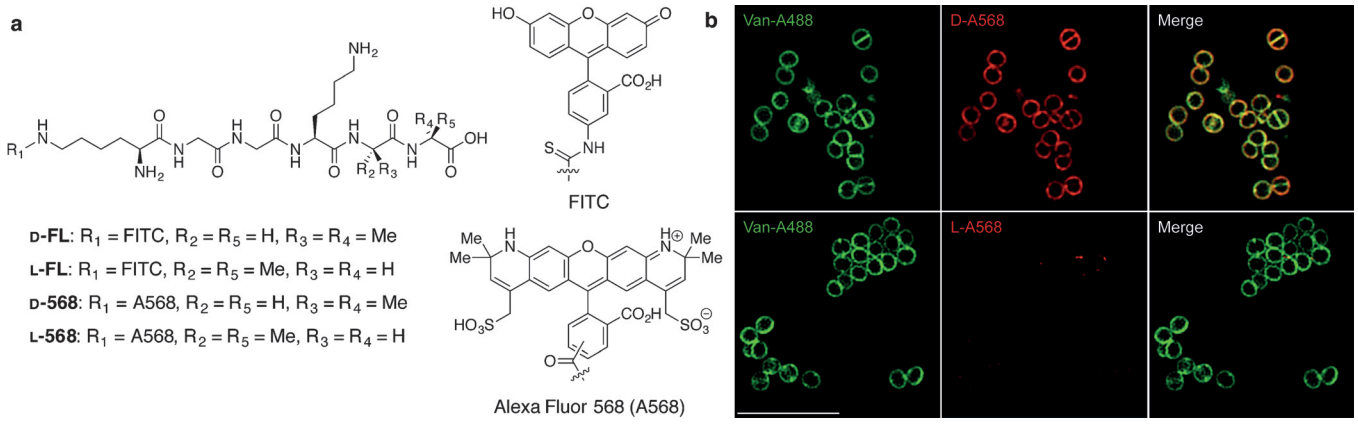

Figure 1. Fluorescent stem peptide mimics (FSPMs) are stereoselectively incorporated into the cell wall of *S. aureus*. a) Chemical structures of probes: D-FL and L-FL; D-A568 and L-A568 b) Representative SIM images of *S. aureus* Newman labeled for 1 hour with D-A568 (top panels) or L-A568 (bottom panels). Both samples were treated with Van-A488, which stains the entire cell wall of *S. aureus* (left panels). Scale bar: 5 μm.

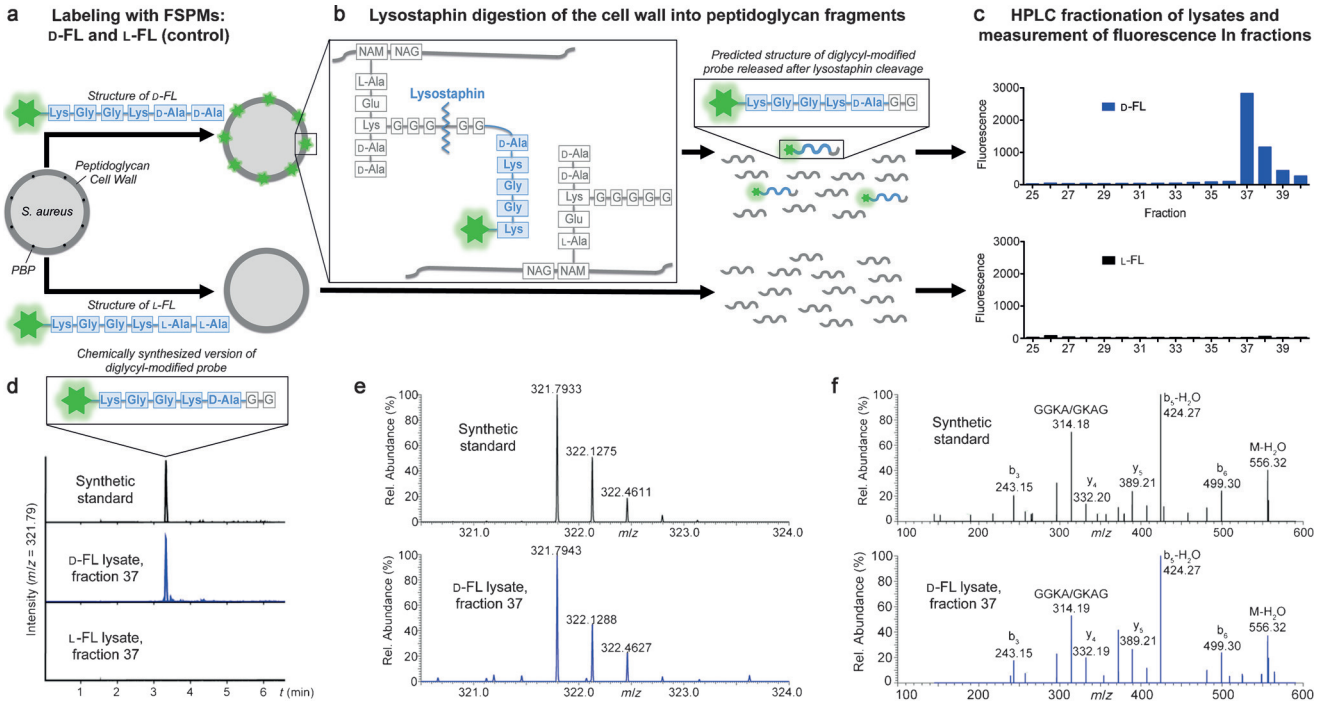

Figure 2. FSPMs are covalently attached to *S. aureus* PG at the site of transpeptidation. a) *S. aureus* Newman WT was treated with 1 mM D-FL or L-FL for 2 h. b) Bacteria were then digested with lysostaphin. c) The resulting lysates were fractionated by HPLC, and the fractions were assayed for fluorescence. d) Fraction 37 from the D-FL lysate and the corresponding fraction from the L-FL lysate were selected for analysis by LC/MS to identify the presence of the diglycyl-modified probe (middle and bottom panels). A peptide with the predicted structure of the modified probe (FITC-KGGKA*GG, m/z of triply charged ion = 321.79) was synthesized and analyzed by LC/MS to provide a standard (top panel). e) High-resolution MS spectra revealed that a compound recovered from fraction 37 of the D-ala lysate (bottom panel) is of an identical isotopic distribution to the synthetic standard peptide (top panel). The triply charged ion of the target peptide FITC-KGGKA*GG is again shown here. f) MS/MS spectra gated on the mass of the target peptide minus FITC (KGGKA*GG, m/z = 574.33) verify that the compound from the D-FL lysate (bottom panel) has the predicted amino acid sequence and is identical to the synthetic standard peptide (top panel). Note: the MS/MS sequence of the recovered compound lacked the FITC moiety, but since the identical pattern was seen for the synthetic standard, we conclude that the relatively weak isothiocyanate bond linking fluorescein to the peptide is simply cleaved during ionization in both cases. A* denotes D-Ala.

In order to establish that FSPMs are covalently attached at the site of endogenous crosslinking (i.e., the bridge peptide), we employed a mass-spectrometry-based experimental strategy. First, *S. aureus* was labeled with either D-FL or L-Fl as shown in Figure 2a. We next treated these bacteria with lysostaphin, a Gly–Gly endopeptidase that cleaves within the pentaglycine bridge peptide.[19] We reasoned that if D-FL were covalently conjugated to the N-terminus of this peptide by a PBP, then lysostaphin treatment should liberate a probe derivative linked to two additional glycines derived from the bridge (Figure 2b). To ascertain whether this diglycyl-modified probe was indeed released after enzymatic cleavage, we fractionated each lysate by HPLC and assayed for fluorescence in individual fractions—an indicator for the presence of the FITC-conjugated peptide (Figure 2c). We did indeed observe fluorescence in fraction 37 and several contiguous fractions from the D-FL-treated lysate, while none appeared in the L-FL control sample.

To verify that the fluorescence in fraction 37 was in fact attributable to the predicted probe derivative, we analyzed the fraction by using liquid chromatography/mass spectrometry (LC/MS). As shown in Figure 2d, we were able to identify a mass corresponding to the diglycyl-modified probe, and furthermore found that it eluted at the same retention time as a chemically synthesized standard peptide: (FITC)–(L-Lys)–(Gly)–(Gly)–(L-Lys)–(D-Ala)–(Gly)–(Gly). No such molecule was found in the corresponding fraction from the L-FL-treated lysate, confirming that the recovered compound is not a naturally occurring species in the cell wall, but rather a product of stereoselective FSPM incorporation. High-resolution MS provided further evidence that the recovered compound was of the same mass as the synthetic standard (Figure 2e) and tandem MS/MS fragmentation analysis was used to confirm the peptide sequence. Taken together, these data show that a glycine-modified derivative of FSPM is released from the cell wall of *S. aureus* treated with D-FL (and not L-FL) upon cleavage of the bridge peptide—strong evidence that FSPMs are covalently and stereoselectively incorporated into the cell wall at the site of crosslinking.

We next sought to identify which PBP isoforms might be responsible for FSPM incorporation in *S. aureus*. PBP1 and PBP2 have essential transpeptidase function and therefore cannot be deleted; however, PBP3 and PBP4 are nonessential so their role in probe labeling could be interrogated directly using deletion mutants.[9,15b] Using this approach, we found that PBP4 is essential for incorporation of D-FL (Figure 3a). To confirm the critical role of PBP4 in probe incorporation, we also showed that labeling is completely abrogated by the

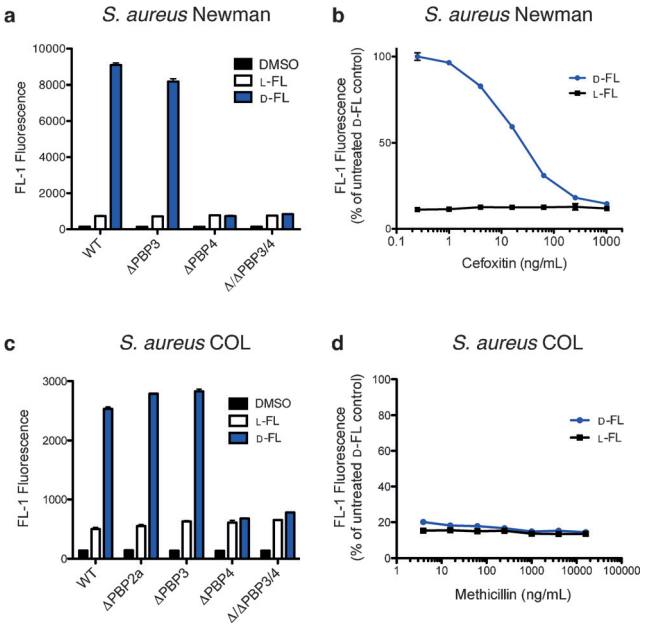

Figure 3. FSPMs are incorporated into *S. aureus* peptidoglycan by PBP4. a) *S. aureus* Newman PBP mutant strains were grown to log phase, labeled with 1 mM D-FL, L-FL, or DMSO vehicle control for 2 h, and analyzed by flow cytometry. b) WT *S. aureus* Newman was pretreated with cefoxitin for 1 h, labeled with probes in the presence of antibiotic for 2 h, and analyzed by flow cytometry. Results shown represent percent values relative to an untreated D-FL sample. c) *S. aureus* COL PBP mutant strains were grown to log phase, labeled with 1 mM D-FL, L-FL, or DMSO vehicle control for 2 h, and analyzed by flow cytometry. d) ΔPBP4 *S. aureus* COL was pretreated with different concentrations of methicillin for 1 h and then labeled with probes for 2 h. Representative flow cytometry experiments performed in technical triplicate are shown; error bars represent SEM.

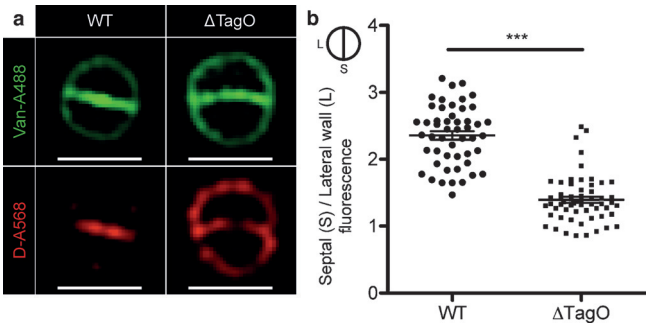

Figure 4. PBP4 activity is restricted to the septum in *S. aureus* in a WTA-dependent manner. WT (left) and ΔTagO (right) *S. aureus* Newman were pulse-labeled for 7.5 min with D-A568 and stained with Van-A488. a) Representative SIM images are shown. b) Fluorescence intensities of the septum and lateral wall in individual cells were assessed from unprocessed wide-field images, and the ratios of each cell are plotted here. Scale bars: 1 μm. Error bars represent SEM; *** indicates a P value < 0.001.

PBP4-specific β-lactam cefoxitin (Figure 3b).[20] Finally, to test the ability of PBP2a to recognize FSPMs, we assessed probe labeling in the MRSA strain COL. As in Newman, PBP4 was entirely responsible for D-FL incorporation in COL strains (Figure 3c); we found no role for PBP2a in probe labeling, even in the presence of concentrations of methicillin known to inhibit the transpeptidase domains of PBP1-4 and elicit PBP2a activity (Figure 3d).[20]

Having characterized FSPM incorporation biochemically, genetically, and pharmacologically, we next sought to use the probe to determine the subcellular localization of PBP4 activity in *S. aureus*. To this end, we performed pulse-labeling studies with D-A568, imaging cells after incubation with probe for 7.5 min (ca. 15% of the organism's 45 min doubling time; Figure S7), which was the earliest timepoint at which labeling was observable. Using this approach, we discovered that PBP4 activity was largely restricted to the septum in dividing cells (Figure 4a, left), consistent with reports of the enzyme's localization.[21]

Based on previous studies showing that PBP4 is recruited to the septum by WTA intermediates, we next hypothesized that probe labeling would redistribute from the crosswall in a WTA-null strain.[13] Indeed, in the TagO mutant, which is deficient in WTA synthesis,[13] we observed just such a redistribution by SIM imaging (Figure 4a, right). Then, using unprocessed wide-field images, we were able to quantify this redistribution as a decrease in the ratio of septal to lateral wall fluorescence (Figure 4b).[13] As in the WT strain, labeling of the TagO mutant was stereoselective, ruling out the possibility that the change in labeling pattern was simply due to global sticking of probe to a cell wall denuded of WTA (Figure S8). We also observed no labeling in the PBP4 mutant, thus demonstrating the specificity of D-A568 for PBP4 (Figure S9). Finally, to confirm that PBP4 is exclusively responsible for probe incorporation even in the absence of WTA, we performed labeling experiments in the TagO mutant in the presence of the PBP4 inhibitor cefoxitin, and in the PBP4 mutant in the presence of the TagO inhibitor tunicamycin (Figure S10).[22] Indeed, labeling was completely abrogated by both pharmacological and genetic depletion of PBP4 activity in cells lacking WTA, demonstrating that PBP4 is required for probe incorporation, regardless of WTA synthesis. Taken together, these data show that PBP4-mediated crosslinking localizes to the septum in *S. aureus* in a WTA-dependent fashion.

In this study, we have presented a new set of activity-based probes for interrogating the crosslinking reaction in vivo. We first demonstrated that FSPMs are covalently incorporated into peptidoglycan by PBP4 in *S. aureus*, and then exploited this specificity to show that PBP4 activity is recruited to the septum in a WTA-dependent manner.

The specificity of FSPMs for PBP4 was rather unexpected, given that all staphylococcal PBPs recognize the same tripeptide motif and should therefore, in theory, be able to incorporate the probe. One explanation for this finding is that PBP1 and PBP2 may exclusively recognize stem peptide present on lipid II, performing crosslinking concurrently with transglycosylation at the septum—a hypothesis consistent with the roles PBP1 and PBP2 play early in the process of PG biosynthesis.[10b,14] In contrast, PBP4, which mediates "secondary crosslinking" (a later stage of PG maturation in which the staphylococcal cell wall acquires its high degree of crosslinking), may recognize free stem peptides present on

PG monomers already incorporated into the sacculus, which the probe is better able to mimic and/or compete with.

Although unanticipated, the isoformic selectivity of FSPMs proved highly useful as it allowed us to interrogate the spatial and temporal properties of a single, defined genetic target (PBP4) in its native cellular setting. Using this approach we showed that PBP4 activity localizes to the septum during cell division, a finding that follows from the work of Pereira et al. and Atilano et al.,[13,21] who first demonstrated the septal localization of PBP4 using fluorescent-protein (FP)-tagged enzymes. With our activity-based probe technology, we were able to both confirm and extend these results, as we showed that PBP4 is not only present at the septum, but that it is active there as well. Recently, we utilized FSPMs to demonstrate that PBP4 is also active outside of the septum, as it appears to redistribute to the peripheral wall between divisions to continue crosslinking.[23] Together, these novel findings illustrate the value of FSPM labeling as a complement to the classic PBP-FP fusion approach in elucidating the spatiotemporal patterns of PBP activity. We believe these probes, together with complementary chemical reporters such as FDAAs,[3a,6,24] will continue to offer valuable insights into the mechanisms of PG biosynthesis, fundamentally advancing our understanding of the bacterial cell wall.

Keywords: bacteria · biosensors · crosslinking · fluorescent probes · peptidoglycans

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