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Chemical Probes Reveal an Extraseptal Mode of Cross-Linking in *Staphylococcus aureus*

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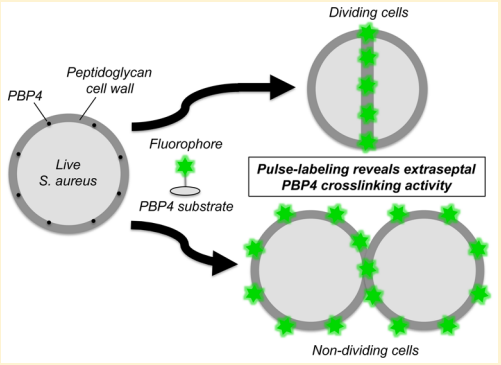
Supporting Information

ABSTRACT: *Staphylococcus aureus* is an important human pathogen and a model organism for studying cell wall synthesis in Gram-positive cocci. The prevailing model of cell wall biogenesis in cocci holds that peptidoglycan synthesis (i.e., transglycosylation and cross-linking) is restricted spatially to the septal cross-wall and temporally to cell division. Previously, we developed a method for visualizing cross-linking in *S. aureus* using fluorescently tagged mimics of the endogenous substrate of penicillin-binding proteins (PBPs). These probes are incorporated into the cell wall of *S. aureus* specifically by PBP4, allowing localization of the enzyme's cross-linking activity *in vivo* with precise spatial and temporal resolution. Here, using this methodology, we have discovered that PBP4 is active not only at the septum, but unexpectedly at the peripheral wall as well. These results challenge the long-held belief that peptidoglycan synthesis is restricted to the septum in spherical bacteria, and instead indicate the presence of two spatiotemporally distinct modes of cross-linking in *S. aureus*: one at the septum during cell division, and another at the peripheral wall between divisions.



INTRODUCTION

The Gram-positive bacterium, *Staphylococcus aureus*, is an important human pathogen, responsible for a rapidly growing epidemic that claims approximately 20 000 lives and 15 billion healthcare dollars each year in the United States alone.[1−3] *S. aureus* also serves as a model organism for cell wall biogenesis in spherical bacteria.[4] This synthetic process is mediated by a class of enzymes called penicillin binding proteins (PBPs), which catalyze three critical reactions (Figure 1 and Supporting Information Figure S1): (i) transglycosylation, the insertion of lipid II monomers into growing peptidoglycan (PG) polymers; (ii) transpeptidation, the covalent cross-linking of neighboring PG strands, which increases the tensile strength of the sacculus; and (iii) carboxypeptidation (a reaction that is absent in *S. aureus*), the cleavage of D-alanine residues from the peptidoglycan stem peptide, which regulates the magnitude of cell wall cross-linking. Methicillin-sensitive *S. aureus* (MSSA) expresses four PBPs (PBP1−4), while resistant strains (MRSA) express an additional isoform, PBP2a, which has very low affinity for β-lactams and therefore allows continued transpeptidation in the presence of these drugs.[4]

Cell wall synthesis has long been thought to be restricted to the septum in *S. aureus* and other cocci.[5] This theory gained support from the elegant pulse labeling studies of Pinho and Errington, and later Turner et al., who showed that insertion of new cell wall material, that is, lipid II, occurs predominantly at the cross-wall.[6,7] Further evidence came from localization studies of staphylococcal PBPs, which display a predominantly septal pattern of expression. Indeed, the two essential high molecular weight (HMW) PBPs in *S. aureus*, PBP1 and PBP2, have both been shown to localize to the septum,[8−10] as has PBP4, *S. aureus*' lone low molecular weight (LMW) PBP.[11,12]

PBP4 plays a number of roles in the physiology and pathogenesis of *S. aureus*. For instance, it is responsible for the unusually high degree of cross-linking characteristic of the staphylococcal cell wall.[13−16] PBP4 is able to produce this effect because, unlike most LMW PBPs, it does not function as a carboxypeptidase *in vivo*. Rather, it possesses robust transpeptidase activity,[17−20] acting late in the process of peptidoglycan maturation[15] to produce a sacculus that is 80% cross-linked (compared to 26% in *Bacillus subtilis*, another Gram-positive model organism).[13,21]

PBP4 also contributes to the clinical burden of *S. aureus*, as it functions as part of a intricate genetic network together with other PBPs (including PBP2a, the key resistance determinant)[15] and enzymes responsible for the synthesis of surface glycopolymers called wall teichoic acids (WTAs)[22−24] to promote resistance to methicillin and other β-lactam antibiotics in community-acquired MRSA.[16,17,25,26] The development of drug resistance in *S. aureus* is largely responsible for the clinical and financial burden imposed by this pathogen, and therefore represents an urgent topic of study.

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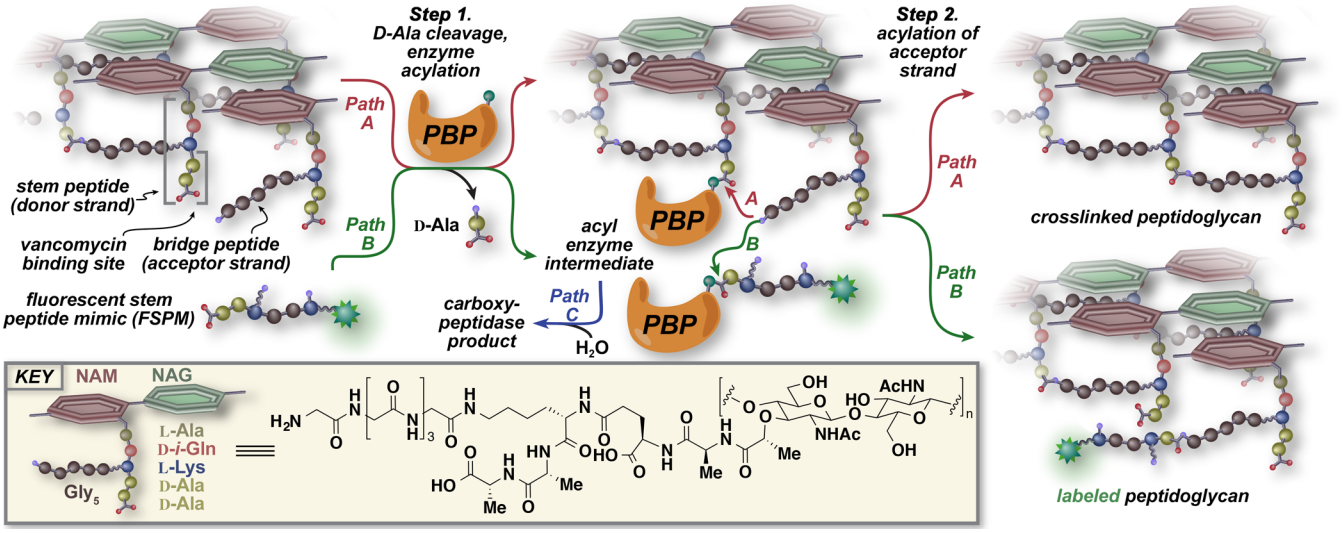

Figure 1. Schematic representation of cross-linking, carboxypeptidation, and FSPM incorporation into the cell wall of *S. aureus*. Two paths are depicted: Path A (red) outlines endogenous PBP-mediated cross-linking of peptidoglycan strands, while Path B (green) illustrates the mechanism of FSPM incorporation. In Path A, the transpeptidase domain of a PBP recognizes the C-terminal terminal L-Lys-D-Ala-D-Ala motif within the stem peptide (donor strand) and cleaves the C-terminal D-Ala residue to form an acyl-enzyme intermediate (Step 1). This intermediate is then captured by the bridge peptide (acceptor strand) to furnish a covalent bond between the penultimate D-Ala of the stem peptide and the N-terminal glycine of the bridge peptide, that is, a cross-link (Step 2). In Path B, FSPMs serve as mimics of the donor strand, producing the unique acyl-enzyme intermediate shown here. The acceptor strand then attacks this intermediate, leading to incorporation of a fluorescent label into peptidoglycan in lieu of a natural cross-link. In Path C, the carboxypeptidase domain of a PBP recognizes the C-terminal terminal L-Lys-D-Ala-D-Ala motif within the stem peptide (donor strand), cleaving the C-terminal D-Ala residue to form an acyl-enzyme intermediate. This intermediate is then captured by a water molecule acceptor to produce a tetrapeptide stem. Abbreviations: D-Ala, D-alanine; FDAA, fluorescent D-amino acid; FSPM, fluorescent stem peptide mimic; Gly, glycine; D-Gln, D-glutamine; L-Ala, L-alanine; Lys, L-lysine; NAM, *N*-acetylmuramic acid; NAG, *N*-acetyglucosamine; PBP, penicillin-binding protein.

One hypothetical mechanism for how PBP4 contributes to β-lactam resistance derives from the work of Atilano et al., who showed that inhibition of WTA synthesis induces PBP4 redistribution from the septum to the "lateral wall" (Figure 2c, ii) in dividing cells.[12] Concomitantly, WTA inhibition leads to a profound decrease in PG cross-linking and restores sensitivity to β-lactams.[22,27] Thus, taken together, these findings have led to the suggestion that the subcellular localization of PBP4 may represent a critical determinant of PG metabolism and antibiotic resistance in *S. aureus*.

With this hypothesis in mind, we set out to characterize the localization patterns of PBP4 activity in *S. aureus* in detail. To do this we utilized a class of activity-based probes for PBP4 previously characterized by our group. These probes, called fluorescent stem peptide mimics (FSPMs, Figure 1 and Scheme 1), are fluorescently tagged analogues of the PG stem peptide, which are recognized by PBP4 and incorporated into the cell wall in place of endogenous cross-links (Figure 1 and Figure S1).[28] Thus, metabolic labeling with FSPMs enables localization of PBP4 transpeptidase activity with high spatial and temporal precision via super-resolution microscopy.[29] Using this system, we previously showed that PBP4 activity is recruited to the septum in dividing cells in a WTA-dependent manner, as predicted by the PBP4 localization studies of Atilano et al.[12,28]

Here we build upon these findings, demonstrating that PBP4 is functional outside the septum as well, mediating a second mode of transpeptidation at the peripheral wall between cell divisions. Also, using a fluorescent derivative of vancomycin, which binds to PBP4's endogenous substrate (the PG stem peptide), we find that PBP4's enzymatic substrate is present at the peripheral wall as well. Together, these data suggest that PBP4 is capable of cross-linking PG both within and outside of the septum in *S. aureus* — a finding that calls into question the long-held assumption that PG synthesis is restricted to the septum in spherical bacteria.

EXPERIMENTAL SECTION

Bacterial Strains and Culture. *S. aureus* Newman WT and ΔPBP4 mutant strains, as well as COL WT and ΔPBP4 mutant strains were generously provided by Ambrose Cheung (Dartmouth School of Medicine).[16] *S. aureus* was cultured in LB broth (Difco, Sparks, MD) and grown at 37 °C with agitation at 200 rpm. Five milliliter liquid cultures were inoculated with single colonies, grown overnight to stationary phase, and then diluted 1:100 and grown 2 h to reach logarithmic phase before addition of probes.

Probe Labeling. For Van-A488 staining experiments, cells were grown to logarithmic phase and then collected by centrifugation at 12 000*g* for 2 min. Cells were washed 3× in ice-cold PBS before staining with Van-A488 or DL-Van-A488 at 2 μg/mL in PBS for 7 min. Cells were again washed 3× in ice-cold PBS before microscopy or flow cytometry analysis. For FSPM labeling experiments, D-A568 or L-A568 were added to logarithmic phase cultures at 1 mM and grown for indicated times. Cells were then immediately washed 3× with ice-cold PBS and incubated with 2 μg/mL Van-A488 for 7 min in PBS at room temperature. Cells were washed 2× in PBS, fixed in 4% paraformaldehyde (diluted from fresh ampules of 16% solution, Electron Microscopy Sciences, Hatfield, PA), and washed 2× with PBS. Finally, cells were resuspended in PBS, dropped onto 1.5 mm glass coverslips coated with poly-L-lysine (Santa Cruz Biotechnology, Santa Cruz, CA), and air-dried. Coverslips were mounted in ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA) and cured for 24 h before imaging.

Flow Cytometry. Samples were diluted in PBS to a concentration of <5,000 cells/μL and 10,000 events per sample were analyzed on an Accuri C6 flow cytometer (BD, Franklin Lakes, NJ) on medium speed fluidics with a minimum threshold of 40,000 FSC-H. Maximum FSC

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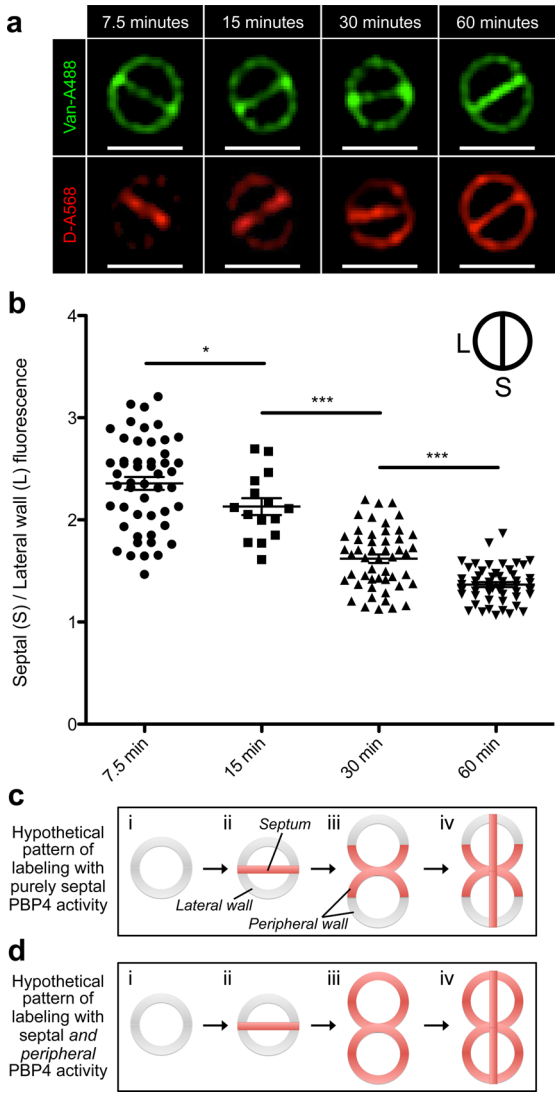

Figure 2. Time course of FSPM labeling reveals uniform signal after only 1.5 cell divisions. WT *S. aureus* Newman was pulse labeled for 7.5, 15, 30, and 60 min with D-A568 and stained with Van-A488 to indicate the PG cell wall. Representative SIM images are shown in (a). Fluorescence intensities of the lateral wall and septum in individual cells were assessed from wide-field images, and ratios for each cell are plotted in (b). Schematics representing the predicted pattern of labeling in a cell exposed to probe for 1.5 generations assuming purely septal PBP4 activity (c) and bimodal septal and peripheral wall activity (d). The lateral wall refers to the nonseptal cell wall during cell division; the peripheral wall refers to the entire cell wall between divisions. Scale bars: 1 μm. Error bars represent SEM; ***$P < 0.001$; *$P < 0.05$.

and SSC gates were set to exclude multicell aggregates (50,000 FSC-A and 50,000 SSC-A). Traces were processed using FlowJo software (Ashland, OR).

Microscopy. Both conventional fluorescence (widefield) and three-dimensional-structured illumination microscopy (3D-SIM) images were acquired using a U-PLANAPO 60×/1.42 PSF, oil immersion objective lens (Olympus, Center Valley, PA) and CoolSNAP HQ2 CCD cameras with a pixel size of 0.080 μm (Photometrics, Tucson, AZ) on the OMX version 3 system (Applied Precision, Issaquah, WA) equipped with 488, 561, and 642 nm solid-state lasers (Coherent, Santa Clara, CA and MPB Communications, Montréal, Quebec). For 3D-SIM imaging, samples were illuminated by a coherent scrambled laser light source that had passed through a diffraction grating to generate the structured illumination by interference of light orders in the image plane to create a 3D sinusoidal pattern, with lateral stripes approximately 0.270 nm apart. The pattern was shifted laterally through five phases and through three angular rotations of 60° for each Z-section, separated by 0.125 nm. Exposure times were typically between 200 and 500 ms, and the power of each laser was adjusted to achieve optimal intensities of between 2,000 and 4,000 counts in a raw image of 16-bit dynamic range, at the lowest possible laser power to minimize photo bleaching. Raw images were processed and reconstructed to reveal structures with 100−125 nm resolution using Softworx software (Applied Precision). The channels were then aligned in *x*, *y*, and rotationally using predetermined shifts as measured using a target lens and the Softworx alignment tool (Applied Precision). For clarity of display, small changes to brightness and contrast were performed on 3D reconstructions. Distribution ratios were calculated for each cell from wide-field images according to a previously reported protocol.[12] Arbitrary line profiles were drawn through the septum and both lateral walls of a dividing cell. The five highest fluorescence intensity values along each profile were selected and then averaged (yielding three average fluorescence values: septum; lateral wall #1; and lateral wall #2). The average septal value was divided by the overall average of the two lateral wall values to yield the final distribution ratio. Data acquisition and analysis was performed by researchers blinded to experimental conditions.

RESULTS

FSPM Labeling Time Course Challenges the Septum-Restricted Model of PG Synthesis. Consistent with our previous results, we found that 7.5 min pulses (~15% of the division time) with the PBP4-specific probe, D-A568 (see Scheme 1 for structure), produces labeling primarily at the septum in dividing *S. aureus*, as observed by SIM imaging (Figure 2a).[28] Using conventional epifluorescence images (Figure 2b), we were able to quantify the relative distribution of label within the cell wall by calculating the ratio of fluorescence at the septum versus the lateral wall. At the 7.5 min time point, this ratio is high, indicating predominant PBP4 activity at the septum.

Next, in order to investigate the dynamics of cross-linking over multiple generations, we tracked the subcellular distribution of FSPM labeling after longer pulses (15−60 min, Figure 2a,b). Over time, we observed that cell wall labeling became more uniform, reflected by a progressive decrease in distribution ratio, eventually reaching uniformity at a ratio of ~1.5 (which is greater than 1 due to the thickness of the septum relative to the lateral walls, as observed previously).[12] These observations are consistent with a model in which newly synthesized, probe-labeled PG gradually replaces old, unlabeled PG.

However, we were surprised by the speed at which FSPM labeling reached uniformity. Assuming a model in which PBP4 is active exclusively at the septum (depicted in Figure 2c), we expected that uniformity would only be reached after several divisions (>2 h), not 60 min (~1.5 generations). At the 15, 30, and 60 min time points, we also expected to see distinct gaps in labeling at the lateral walls (e.g., Figure 2c, iii and iv), reflecting areas of old, "peripheral cell wall" (Figure 2c, iii) that could not be labeled with FSPM. Instead, however, we observed continuous labeling that simply intensified over time. Together these results seemed inconsistent with the notion that PBP4 activity is restricted to the cross-wall.

PBP4 is Active at the Peripheral Wall in Nondividing Cells. To explain these findings, we hypothesized that PBP4 redistributes after cell division to perform transpeptidation

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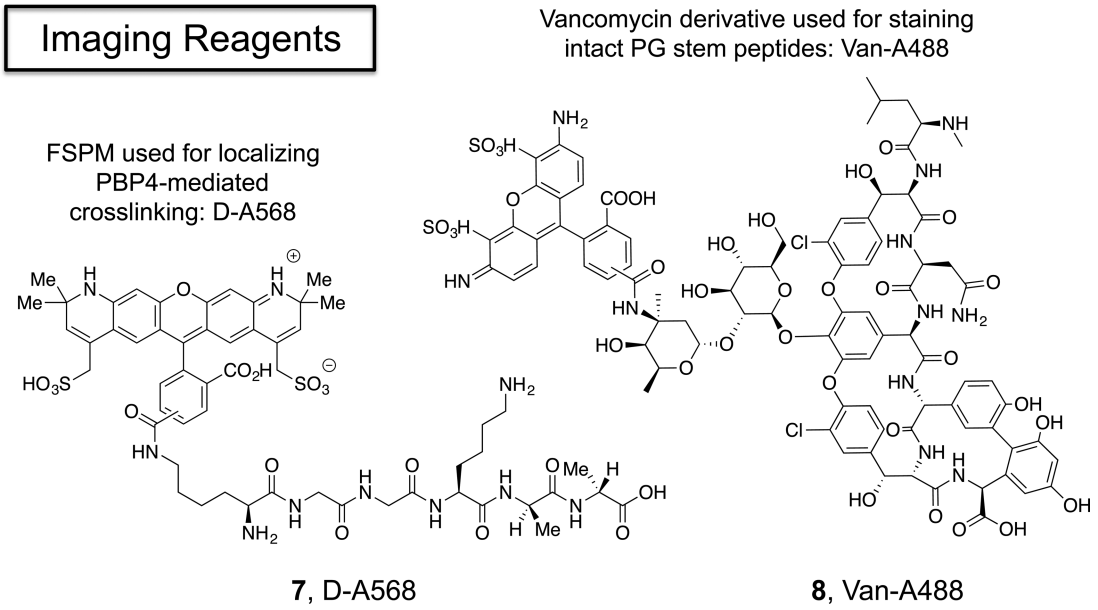
Scheme 1. Chemical Structures of Synthetic Imaging Reagents Used in This Study: D-A568 and Van-A488[a]



[a]Abbreviations: FSPM, fluorescent stem peptide mimics; PBP4, penicillin-binding protein 4; PG, peptidoglycan.

circumferentially at the peripheral wall (Figure 2d, (iii). To test this theory, we briefly pulsed logarithmic phase *S. aureus* (7.5 min) with D-A568 and imaged nonseptating cells. Indeed, there was significant labeling of the peripheral wall in these cells (Figure 3a, top panels), suggesting that PBP4 is in fact active outside of the septum.

To rule out the possibility that circumferential labeling results from "sticking" of the probe to the cell wall, we performed identical labeling experiments substituting D-A568 with a diastereomeric control probe, L-A568 (Figure 3a, bottom panels). Minimal fluorescence was observed under these conditions. In addition, labeling experiments performed in an isogenic PBP4 knockout mutant (Figure S2) also produced negligible signal. Taken together, these results show that peripheral D-A568 probe labeling is attributable not to nonspecific sticking, but rather to enzymatic incorporation mediated by PBP4.

We next sought to rule out the possibility that the signal observed at the peripheral wall is the result of "redistribution" of probe-modified PG away from a septal site of incorporation during the labeling period. To do so, we needed a marker for "old PG" not derived from the septum during the course of the pulse labeling experiment. To this end, we employed a pulse-chase strategy in which cells were first labeled uniformly with D-A647 (an Alexa Fluor 647 conjugate of FSPM) for 2 h, and then allowed to grow in the absence of probe for more than one generation (60 min) (Figure 3b). As expected, this procedure produced D-A647-labeled "caps" of old PG in nondividing *S. aureus* doublets that indicate peripheral wall (Figure 3c, Supporting Information movies). With this region of the sacculus clearly demarcated, we then pulsed with D-A568 for 7.5 min to label sites of newly synthesized PG cross-links with an orthogonal fluorophore. As shown in Figure 3c, D-A568 labeling overlaps with the D-A647 peripheral wall marker, demonstrating that PBP4 is active outside the septum, where it modifies old PG.

Endogenous PBP4 Substrate (PG Stem Peptide) Is Also Present at the Peripheral Wall. A critical caveat of these experiments is that FSPM labeling only indicates locations of biochemically active PBP4—i.e. regions where PBP4 is capable of recognizing and attaching an *exogenous* donor substrate to an acceptor bridge peptide within the cell wall. Thus, while our results confirm that PBP4 is active at the peripheral wall, and that acceptor peptide substrates are available there, they do not provide direct evidence that PBP4-mediated cross-linking occurs at this site. To assert this point more confidently, we therefore sought to demonstrate that PBP4's endogenous substrate, the C-terminal L-Lys-D-Ala-D-Ala motif within the stem peptide, is also located at the peripheral wall.

To define the subcellular localization of this motif, we made use of the Van-A488 imaging reagent, which binds to the terminal D-Ala-D-Ala moiety (Figure 1 and Figure S1a). Before proceeding with localization experiments, however, we first sought to establish that Van-A488 is indeed capable of recognizing PBP4 substrate. To achieve this, we took an indirect approach, comparing Van-A488 staining in WT and PBP4 mutant cells. We reasoned that WT *S. aureus* would have a lower abundance of PBP4 substrate due to enzyme-mediated consumption, while in the PBP4 mutant, more substrate would remain intact. Therefore, if Van-A488 is able to bind PBP4 substrate, staining should be stronger in the ΔPBP4 mutant. Indeed, flow cytometry quantification of Van-A488 staining did reveal higher labeling in the PBP4 mutant in both Newman and COL strains (Figure 4a), thus confirming that the reagent is able to identify PBP4 substrate within the cell wall.

To show that the observed increases in staining in the ΔPBP4 mutant were not simply due to physical alterations in the cell wall that promoted D-Ala-D-Ala-independent sticking of Van-A488, we stained with a Van-A488 derivative: DL-Van-A488, which lacks an *N*-methyl leucine residue necessary for binding to the D-Ala-D-Ala motif.[30] This compound thus recapitulates the nonspecific binding properties of Van-A488, but does not recognize the stem peptide. As shown in Figure 4a, DL-Van-A488 staining did not change significantly in the absence of PBP4, confirming that the increased Van-A488

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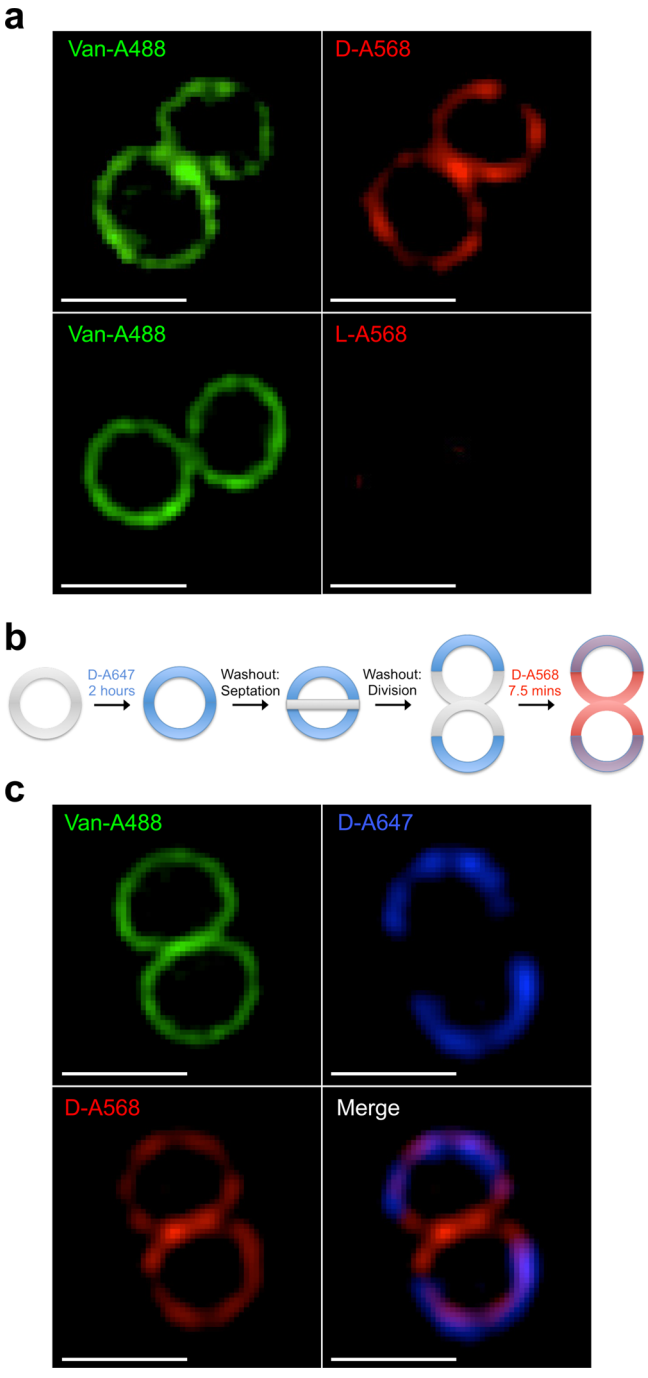

Figure 3. PBP4 cross-linking activity occurs at the peripheral wall in nondividing cells. (a) WT *S. aureus* Newman was pulse labeled for 7.5 min with D-A568 (top panels) or L-A568 (bottom panels) and stained with Van-A488. Representative SIM images of nonseptating doublets are shown. (b) Schematic representing the design of the pulse-chase experiment. (c) WT *S. aureus* Newman was labeled for 2 h with D-A647. Probe was then washed out and cells were allowed to grow for 1 h before being pulse labeled for 7.5 min with D-A568 and stained with Van-A488. Representative SIM images of a nonseptating doublet are shown. Scale bars: 1 μm.

labeling in the ΔPBP4 mutants indeed indicates a greater abundance of PBP4 D-ala-D-ala substrate in these strains.

Having established the ability of Van-A488 to bind PBP4 substrate, we utilized it in imaging experiments to characterize substrate localization in nondividing cells. As shown in Figure

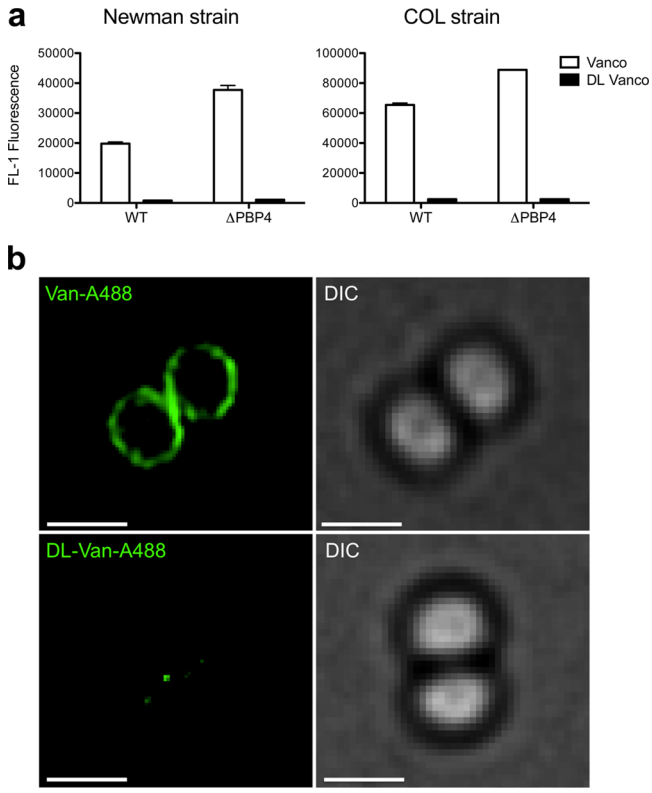

Figure 4. Endogenous PBP4 substrate (PG stem peptide) is present at the peripheral wall. (a) WT and ΔPBP4 *S. aureus* against Newman and COL backgrounds were stained with 2 μg/mL Van-A488 or DL-Van-A488 for 5 min. Total cellular fluorescence was quantitated by flow cytometry. Error bars represent SEM. (b) WT *S. aureus* Newman was stained with 2 μg/mL Van-A488 or DL-Van-A488 for 5 min and imaged by SIM. Representative images of nonseptating cells are shown. Scale bars: 1 μm.

4b, we observed robust circumferential Van-A488 staining in such cells, indicating the presence of PBP4 substrate at the peripheral wall. Notably, this pattern of vancomycin labeling in nondividing *S. aureus* has also been observed by previous investigators,[6] who attributed the finding to the lack of carboxypeptidase activity in *S. aureus*, which allows intact D-Ala-D-Ala motifs to persist throughout the sacculus after division has occurred.[31] This pattern contrasts with observations in other organisms, wherein vancomycin binding is observed predominantly at sites of lipid II insertion, due to the activity of carboxypeptidases that rapidly cleave terminal D-Ala residues after transglycosylation.[32]

Finally, to rule out the possibility that peripheral Van-A488 staining is due to nonspecific sticking of the compound, we stained with the DL-Van-A488 control. As shown in Figure 4b, DL-Van-A488 produced no significant labeling, confirming that Van-A488 staining reliably indicates the presence of D-Ala-D-Ala (and thus intact PBP4 substrate) at the peripheral wall.

DISCUSSION

In this study we have used FSPMs, a set of chemical probes for PBP4 cross-linking activity, to show that the enzyme is functional at the peripheral wall of nondividing *S. aureus*. Implicitly, labeling at this extra-septal location also demonstrates the presence of pentaglycine acceptor peptide, since the probe is known to be conjugated specifically to this PG motif.[28] In addition, we show that a vancomycin-based probe capable of

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binding to PBP4's endogenous donor substrate (the stem peptide) stains the peripheral wall. Thus, although our data do not unequivocally prove that PBP4-mediated cross-linking occurs at the peripheral wall, it appears highly likely based on the colocalization of active enzyme, acceptor substrate, and donor substrate in this location.

Together, these results point to a revised model of transpeptidation in *S. aureus* that involves two modes of cross-linking (Figure 5): one at the septum mediated by PBP1,

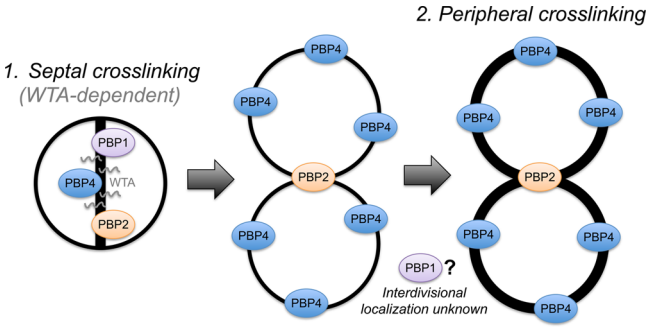

Figure 5. Hypothetical model describing the septal and peripheral modes of cross-linking in *S. aureus*. PBP4 localizes to the septum in a WTA-dependent manner;[12] PBP2 is recruited by its substrate;[8] PBP1 also localizes to septum.[9,10] PBP2 mediates transglycosylation and initial cross-linking events,[8,15,34] while PBP4 increases the degree of septal cross-linking.[15] PBP1 serves an unknown scaffolding role and also carries out a transpeptidation event late in septation that is critical for cell separation.[9,10] PBP4 redistributes circumferentially after cell division to continue PG cross-linking at the peripheral wall (this study). PBP2 remains localized to the former division site.[8] The interdivision localization of PBP1 has not yet been determined.

PBP2, and PBP4 (the latter two perhaps working in a coordinated complex),[15,22] and the other at the peripheral wall mediated exclusively by PBP4 (note: the localization of PBP3 remains unknown). Meanwhile, the insertion of new cell wall material (i.e., lipid II) via PBP2-mediated transglycosylation appears to occur mostly at the septum.[6,7]

These findings beg the question of what functional impact extra-septal cross-linking has on *S. aureus* cell wall physiology. One likely effect is that peripheral PBP4 activity contributes the unusually high degree of cross-linking found in the staphylococcal cell wall.[13−16] Cross-linking at the peripheral wall is perhaps also necessary to offset the degradative action of autolysins that may localize there. Indeed, atomic force and electron micrographs of "old PG" at the peripheral wall reveal a rougher texture compared to new, septal-derived PG, which has a concentric ring-like architecture;[7,33] this postseptation ultrastructural remodeling may be attributable in part to PBP4 activity.

The determinants of PBP4 localization through the cell cycle also remain unclear. Atilano et al. clearly showed that WTA synthesis plays a role in PBP4 recruitment to the septum during division, as it evenly redistributes throughout the cell wall in the absence of TagO, the first enzyme in the WTA synthetic pathway.[12] Previously, we extended this work using FSPM labeling to show that septal PBP *activity* is likewise dependent on WTA biosynthesis.[28] Given that mature WTA are present globally in the PG sacculus, it was proposed that nascent WTA intermediates at the septum recruit PBP4, perhaps through direct physical interaction. An alternative hypothesis suggested by Atilano et al. is that TagO deletion perturbs the metabolism and localization of bactoprenol, the common precursor of both WTA and lipid II. Thus, lipid II may redistribute from the septum in the absence of TagO, allowing PBP4 to disperse as well. Along these lines, it was recently shown that WTA inhibition in *Bacillus subtilis* induces delocalization of lipid II, PBP2a, and PG biosynthesis.[35] The mechanistic basis for PBP4 redistribution to the peripheral wall between divisions also remains unknown; investigations into this phenomenon are currently ongoing in our laboratory.

Through a combination of classic bacteriology and novel chemical biology techniques such as metabolic labeling with the FSPMs used here and the fluorescent D-amino acids described elsewhere,[20,36] we believe these mechanisms will become increasingly clear, deepening our understanding of cell wall physiology and informing the rational development of novel antibacterial therapy.

■ ASSOCIATED CONTENT

Supporting Information

Chemical synthesis, Figures S1 and S2, and supplemental movies. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b02972.

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Notes

The authors declare the following competing financial interest(s): D.A.S. is a paid consultant for Bristol-Myers Squibb.

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