Communication

Peptidoglycan crosslinking preferences of *Staphylococcus aureus* penicillin-binding proteins have implications for treating MRSA

Veerasak Srisuknimit, Yuan Qiao, Kaitlin Schaefer, Daniel Kahne, and Suzanne Walker

J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.7b04881 • Publication Date (Web): 10 Jul 2017

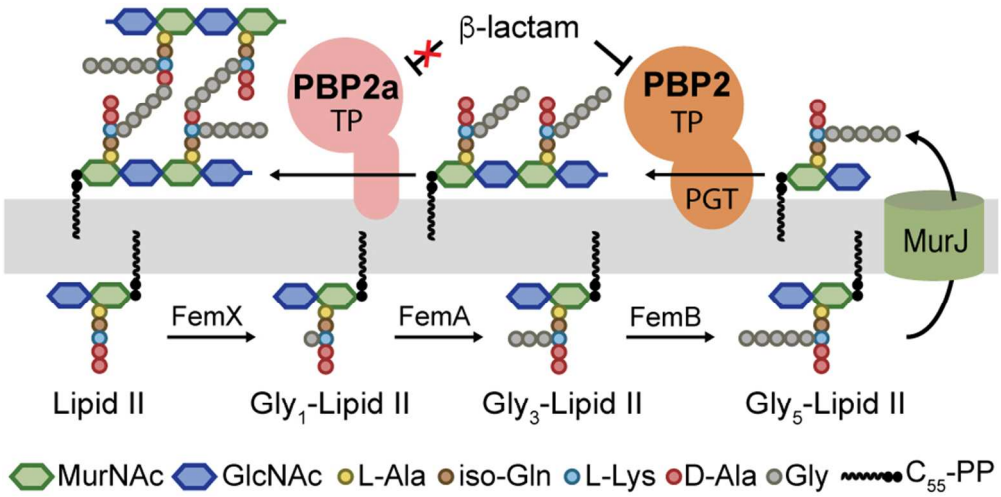


Figure 1. Schematic of peptidoglycan biosynthesis in methicillin-resistant *S. aureus*. Three enzymes, FemXAB, attach five glycines to the lysine side chain of the peptidoglycan precursor Lipid II. FemX is essential but cells can survive without FemA or FemB. After export to the cell surface, Gly_5-Lipid II is polymerized by peptidoglycan glycosyltransferases (PGTs) and the glycan strands are crosslinked by transpeptidases (TPs). PGT domains are found in bifunctional enzymes such as PBP2 (shown), which also contain transpeptidase domains, or in monofunctional enzymes. PBP2a crosslinks glycan strands when native TPs, e.g., as in PBP2, are inhibited by β-lactams.

83x41mm (300 x 300 DPI)

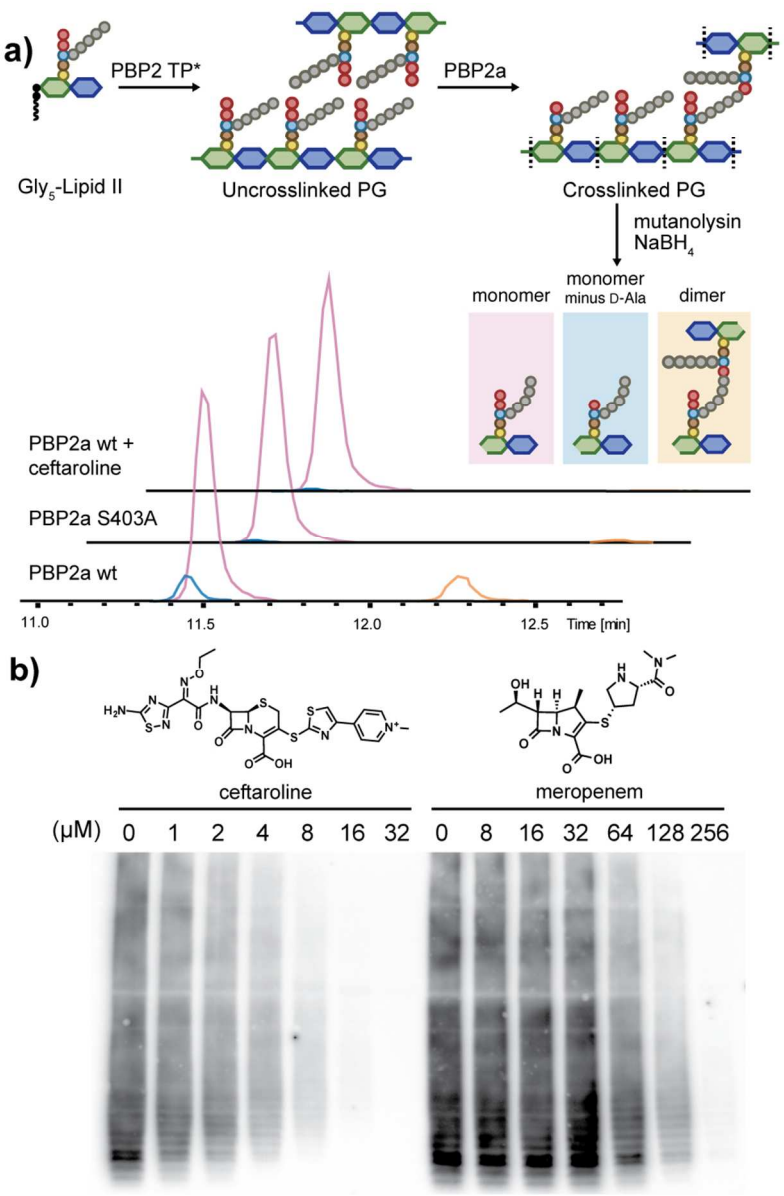


Figure 2. Reconstitution of PBP2a activity and inhibition. (a) Demonstration of PBP2a activity by LC/MS. Extracted ion chromatogram (EIC) for the wild type PBP2a (PBP2a wt) shows both hydrolysis and crosslinked products, but the catalytically inactive mutant (PBP2a S403A) or ceftaroline-treated PBP2a do not. (b) Western blot showing inhibition of PBP2a traspeptidation in the presence of different concentrations of ceftaroline and meropenem. Peptidoglycan fragments incorporate biotin-D-lysine unless PBP2a transpeptidase activity is inhibited (see Methods).

83x125mm (300 x 300 DPI)

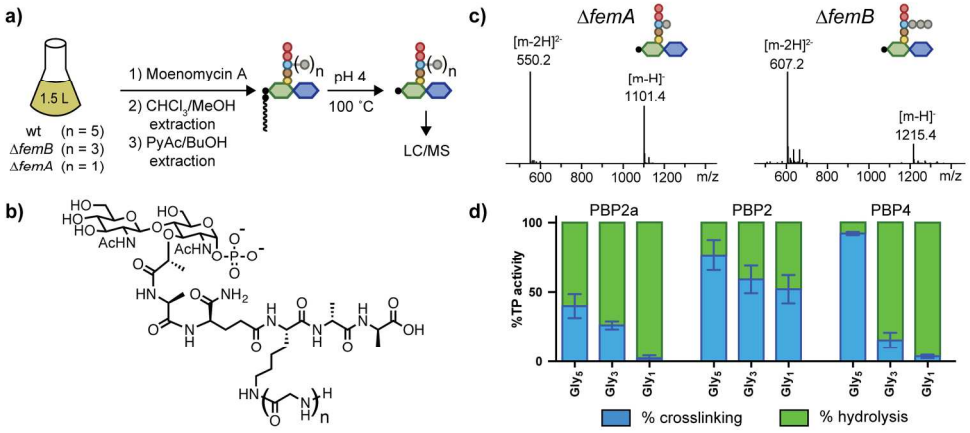
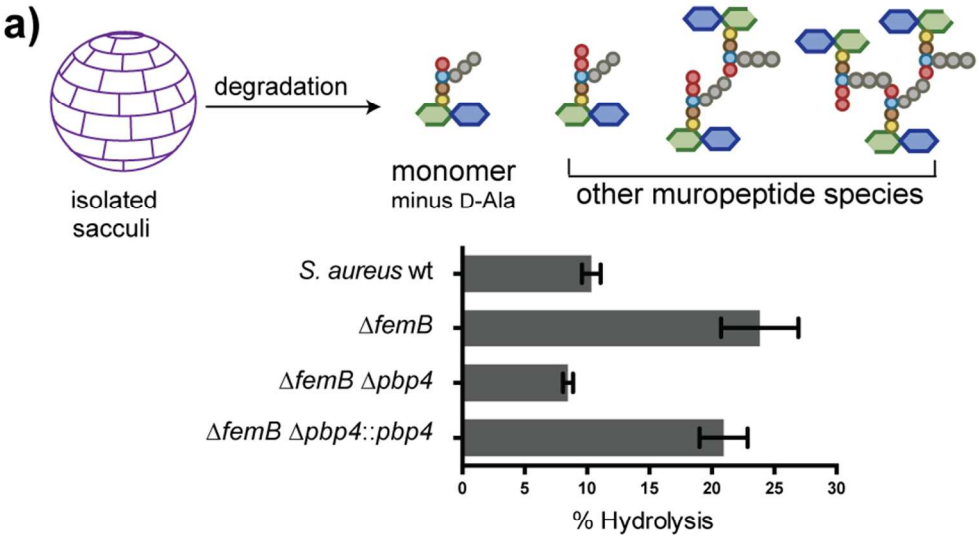


Figure 3. The ratio of crosslinking to hydrolysis for *S. aureus* PBPs depends on both the enzyme and the substrate. (a) Schematic of the Lipid II isolation procedure with removal of the lipid tail for LC/MS analysis. *S. aureus* wt. or mutant strains are treated with moenomycin A, which inhibits PGT activity, to accumulate Lipid II, which is then isolated by a two-step extraction procedure (see Methods). Acidic hydrolysis of the isolated Lipid II removes the undecaprenyl-phosphate chain for LC/MS analysis. (b) Chemical structure of Lipid II monophosphates after acid hydrolysis. (c) LC/MS analysis of delipidated Gly_1- and Gly_3-Lipid II monophosphates isolated from the *ΔfemA* and *ΔfemB* mutants, respectively (also see SI Fig. 4 and 5 for LC/MS/MS). (d) A bar graph showing ratios of crosslinking and hydrolysis activities for PBP2a, PBP2 and PBP4 with Gly_5-, Gly_3-, and Gly_1-peptidoglycan (see Methods and SI Table 1).

178x78mm (300 x 300 DPI)

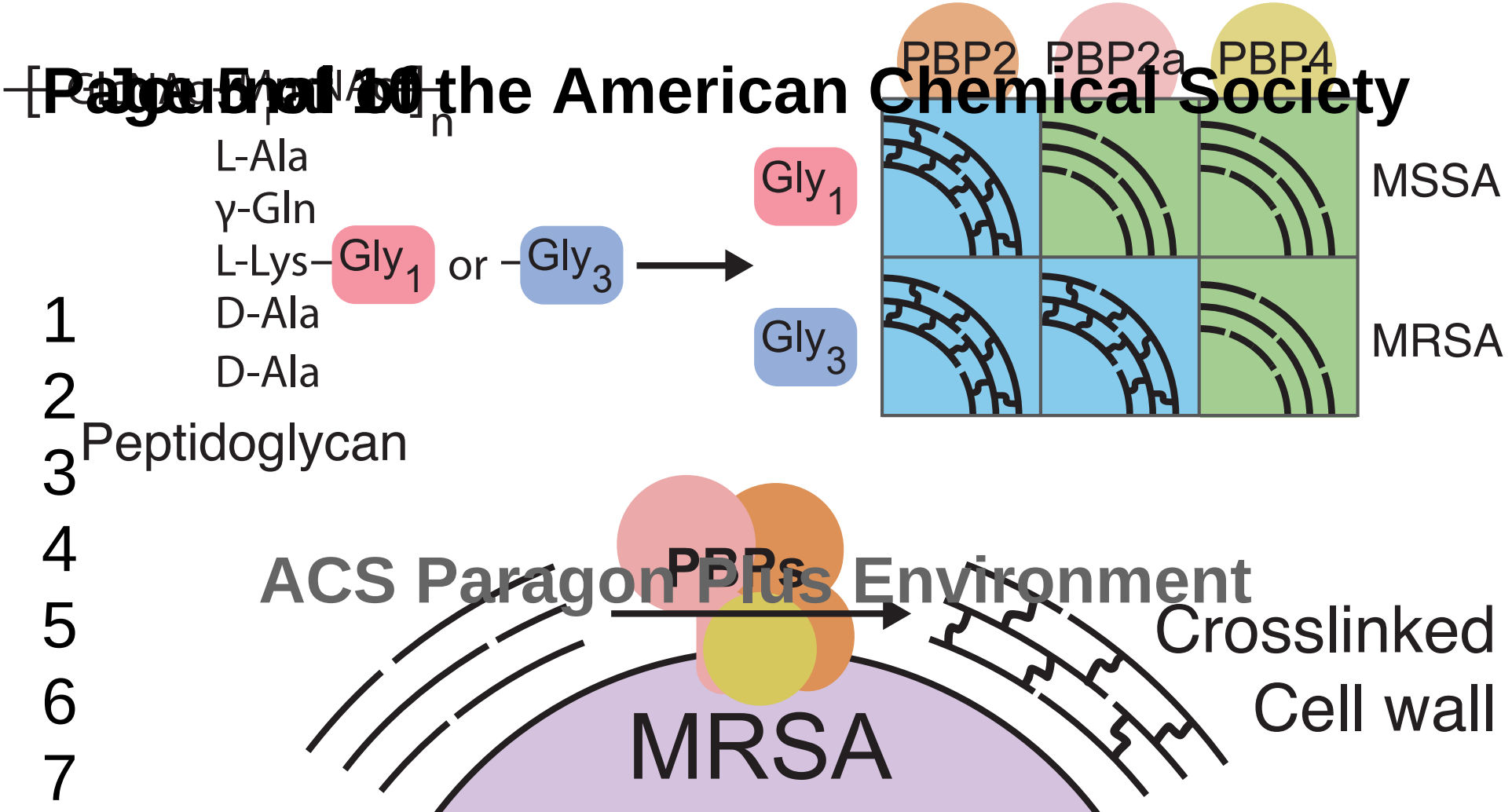


b)

inhibitor (PBP selectivity)	MIC (μg/mL)			
	wt	*ΔfemB*	*mecAΔfemB*	*mecAΔfemA*
ceftaroline (1, 2, 2a, 3)	2	1	2	0.25
piperacillin (1, 2, 3)	1	0.5	4	1
cefoxitin (4 > 2)	2	2	32	1

Figure 4. In cells, Gly_3-peptidoglycan undergoes hydrolysis by PBP4 yet can be crosslinked by PBP2a. (a) *S. aureus* sacculi were degraded and analyzed by LC/MS to determine the extent of D-Ala hydrolysis. The bar graph shows the percentage of hydrolyzed muropeptide compared to other depicted species. Deleting PBP4 reduces hydrolysis. (b) Minimum inhibitory concentrations (MICs) for three different β-lactams against four *S. aureus* strains, with the targeted PBPs for each β-lactam shown in parentheses. The presence of *mecA*, encoding PBP2a, in the *ΔfemB* background increases the MIC of piperacillin and cefoxitin, but not ceftaroline, consistent with the in vitro data that PBP2a can crosslink Gly_3-peptidoglycan. In the *ΔfemA* background, *mecA* does not confer resistance.

83x78mm (300 x 300 DPI)



Peptidoglycan crosslinking preferences of *Staphylococcus aureus* penicillin-binding proteins have implications for treating MRSA

Veerasak Srisuknimit[1‡], Yuan Qiao[1,2‡], Kaitlin Schaefer[1,2], Daniel Kahne[1*], Suzanne Walker[2*]

[1] Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts, 02138, United States

[2] Department of Microbiology and Immunology, Harvard Medical School, Boston, Massachusetts, 02138, United States

Supporting Information Placeholder

ABSTRACT: Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a global public health problem. MRSA strains have acquired a non-native penicillin-binding protein called PBP2a that crosslinks peptidoglycan when the native *S. aureus* PBPs are inhibited by β-lactams. It has been proposed that the native *S. aureus* PBPs can use cell wall precursors having different glycine branch lengths (penta-, tri-, or monoglycine), while PBP2a can only crosslink peptidoglycan strands bearing a complete pentaglycine branch. This hypothesis has never been tested because the necessary substrates have not been available. Here, we compared the ability of PBP2a and two native *S. aureus* transpeptidases to crosslink peptidoglycan strands bearing different glycine branches. We show that purified PBP2a can crosslink glycan strands bearing penta- and triglycine, but not monoglycine, and experiments in cells provide support for these findings. Because PBP2a cannot crosslink peptidoglycan containing monoglycine, this study implicates the enzyme (FemA) that extends the monoglycine branch to triglycine on Lipid II as an ideal target for small molecules that restore sensitivity of MRSA to beta-lactams.

Bacterial cells are encased in a peptidoglycan (PG) matrix that is required for their survival. β-lactam antibiotics target peptidoglycan biosynthesis and they are still widely used due to their broad spectrum and excellent safety profile. β-lactams covalently inactivate the transpeptidase (TP) domains of penicillin-binding proteins (PBPs) and prevent crosslinking of peptidoglycan strands (SI Figure 1).[1] In most organisms, β-lactam resistance is due to expression of β-lactamases that destroy β-lactams before they can inactivate their lethal targets. This form of resistance can be overcome by using a β-lactamase inhibitor in combination with a β-lactam.[1] However, resistance in methicillin-resistant *S. aureus* (MRSA) arises through acquisition of a gene cassette containing *mecA*, which encodes an intrinsically resistant transpeptidase, PBP2a, that crosslinks peptidoglycan when other transpeptidases are inhibited by β-lactams.[2] MRSA is responsible for more than half of deaths due to antibiotic resistant infections in the United States.[3] Strategies to overcome β-lactam resistance in MRSA have focused on developing new β-lactams that inhibit PBP2a, such as ceftaroline and ceftobiprole, and on identifying β-lactam potentiators for use in combination with β-lactams.[2a,4]

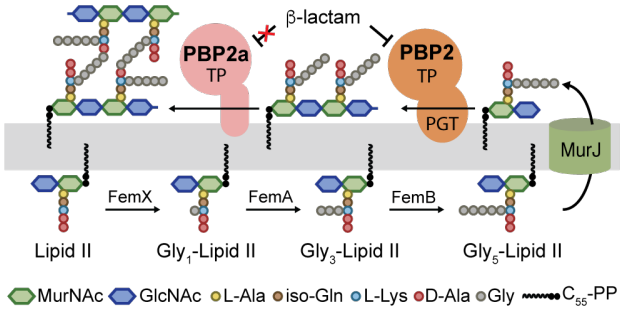


Figure 1. Schematic of peptidoglycan biosynthesis in methicillin-resistant *S. aureus.* Three enzymes, FemXAB, attach five glycines to the lysine side chain of the peptidoglycan precursor Lipid II. FemX is essential but cells can survive without FemA or FemB.[5] After export to the cell surface, Gly_5-Lipid II is polymerized by peptidoglycan glycosyltransferases (PGTs) and the glycan strands are crosslinked by transpeptidases (TPs). PGT domains are found in bifunctional enzymes such as PBP2 (shown), which also contain transpeptidase domains, or in monofunctional enzymes. PBP2a crosslinks glycan strands when native TPs, *e.g.,* as in PBP2, are inhibited by β-lactams.

β-lactam potentiators are compounds that increase the susceptibility of MRSA strains to β-lactams. Genetic studies have suggested over two dozen targets for β-lactam potentiators, including FemA and FemB, which are required for synthesis of the pentaglycine branch on *S. aureus* Lipid II, the peptidoglycan precursor (Figure 1).[5] *S. aureus* Lipid II consists of a lipid-linked disaccharide containing a pentapeptide stem with a pentaglycine branch attached to the lysine of the stem peptide. FemA installs the second and third glycines of this branch, making Gly_3-Lipid II, and FemB installs the fourth and fifth, making Gly_5-Lipid II. Once assembled, Gly_5-Lipid II is flipped to the extracellular surface of the membrane where it is polymerized and crosslinked (Figure 1). *S. aureus* strains lacking *femA* or *femB* are viable, implying that one or more of the native PBPs can crosslink peptidoglycan containing Gly_1- and Gly_3-peptidoglycan.[5a,5b] However, deletion of these genes increases susceptibility to β-lactams even when PBP2a is expressed. Therefore, it has been proposed that PBP2a can only crosslink Gly_5-peptidoglycan.[6]

The crosslinking preferences of *S. aureus* PBPs have not been characterized due to the major challenges involved in obtaining uncrosslinked peptidoglycan substrates bearing different glycine branches. We recently developed a method to obtain Gly_5-Lipid II in large amounts from *S. aureus*, enabling access to Gly_5-peptidoglycan.[7,8] Here we report that it is possible to obtain substantial quantities of Gly_1- and Gly_3-Lipid II from appropriate *S. aureus* mutant strains, which has enabled us to examine the crosslinking preferences of native *S. aureus* PBPs as well as PBP2a.

The transpeptidase activity of PBP2a has not been previously reconstituted. Therefore, we first developed conditions to monitor crosslinking of uncrosslinked glycan strands produced from the native cell wall precursor, Gly_5-Lipid II. Gly_5-Lipid II was incubated with PBP2a and a mutant of PBP2 (PBP2-TP*) that contains an active peptidoglycan glycosyltransferase (PGT) domain but a catalytically inactive transpeptidase domain. Reactions were quenched after 3 h and the polymeric products were treated with mutanolysin and $NaBH_4$ to produce muropeptide fragments for LC/MS analysis (Figure 2a).[9] We detected three major products: monomer (pink peak), hydrolyzed monomer (monomer minus D-Ala, blue peak), and dimer (orange peak). The hydrolyzed monomer and dimer peaks disappeared when PBP2a was genetically inactivated by mutating the active site serine to alanine (Figure 2a, PBP2a S403A; SI Figure 2), confirming that they result from PBP2a activity.[10] Therefore, PBP2a can activate the terminal amide bond in the pentapeptide, resulting in crosslinking if the pentaglycine attacks the covalent intermediate or hydrolysis if water attacks (see SI Figure 1).

We next examined the β-lactam susceptibility profile of PBP2a. Previous studies showed that PBP2a can be inactivated by the fifth-generation cephalosporins, ceftaroline and ceftobiprole, and is weakly inhibited by meropenem, but not other β-lactams.[2a,11] We found that incubation of PBP2a with ceftaroline prior to adding PBP2-TP* and Gly_5-Lipid II abolished formation of the dimer and hydrolyzed monomer peaks in the LC/MS assay (Figure 2a). We also used a PAGE-immunoblot assay to compare inhibitions of transpeptidase activity by these β-lactams (Figure 2b, SI Figure 3).[7] Meropenem inhibited PBP2a, but was about twenty-fold less potent than ceftaroline, consistent with results obtained from competition binding assays for these β-lactams.[11a,12]

We next examined the substrate preferences of PBP2a and two other *S. aureus* PBPs, PBP2 and PBP4. PBP2 is the only PBP in *S. aureus* that contains both a peptidoglycan glycosyltransferase domain and a transpeptidase domain, and it is essential for survival.[13] PBP4, which contains only a transpeptidase domain, is not essential for survival, but is known for installing additional crosslinks in peptidoglycan and has been implicated in β-lactam resistance in some MRSA strains.[14] Using the method previously developed to obtain Gly_5-Lipid II, we isolated Gly_1- and Gly_3-Lipid II from Δ*femA* and Δ*femB S. aureus* strains, respectively (Figure 3a-3c).[7] To investigate crosslinking preferences, we incubated each substrate with either (1) PBP2 TP* and PBP2a, (2) PBP2 wt, or (3) PBP2 TP* and PBP4. For each condition, we quantified the ratio of crosslinked muropeptide and hydrolyzed monomer (minus D-Ala) using LC/MS (Figure 3d). The combined crosslinking and hydrolysis activities were approximately constant across different substrates for each enzyme, showing that formation of the acyl-enzyme intermediate was not sensitive to the length of the glycine branch; however, the proportion of activity due to crosslinking versus hydrolysis varied greatly. PBP2a preferred Gly_5- peptidoglycan, but also crosslinked Gly_3-peptidoglycan; however, it hydrolyzed Gly_1-peptidoglycan. PBP2 crosslinked all three substrates. PBP4, the low-molecular weight PBP, showed the most dramatic switch in activity as the glycine branch was truncated.

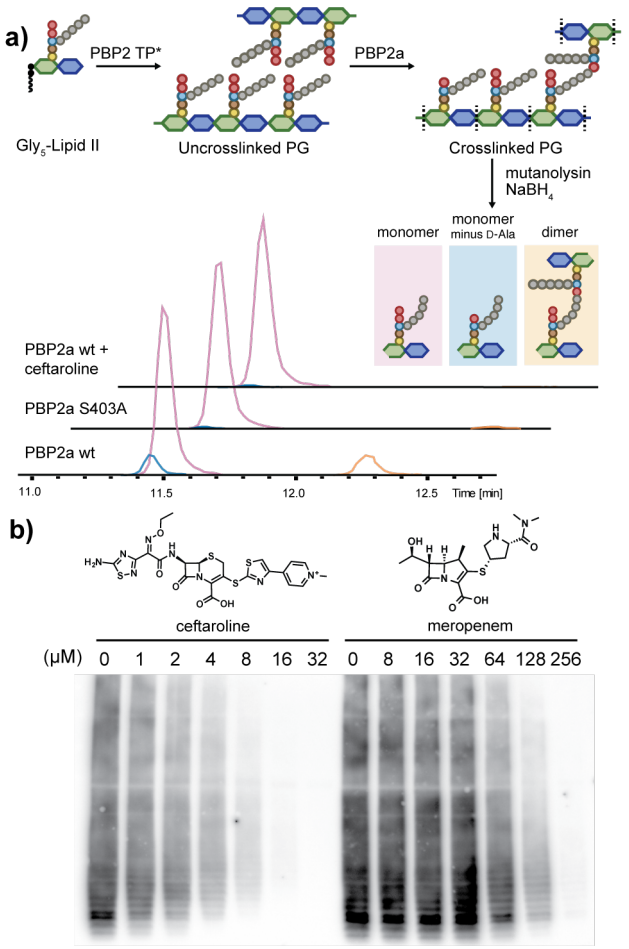


Figure 2. Reconstitution of PBP2a activity and inhibition. (a) Demonstration of PBP2a activity by LC/MS. Extracted ion chromatogram (EIC) for the wild type PBP2a (PBP2a wt) shows both hydrolysis and crosslinked products, but the catalytically inactive mutant (PBP2a S403A) or ceftaroline-treated PBP2a do not. (b) Western blot showing inhibition of PBP2a traspeptidation in the presence of different concentrations of ceftaroline and meropenem. Peptidoglycan fragments incorporate biotin-D-lysine unless PBP2a transpeptidase activity is inhibited (see Methods).

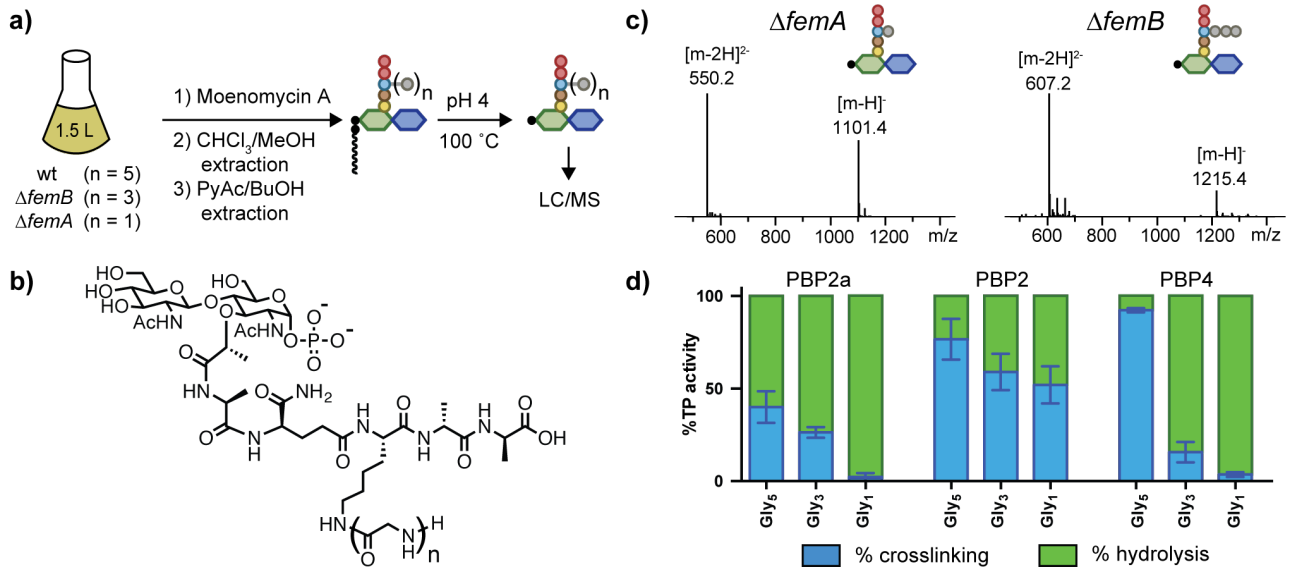


Figure 3. The ratio of crosslinking to hydrolysis for *S. aureus* PBPs depends on both the enzyme and the substrate. (a) Schematic of the Lipid II isolation procedure with removal of the lipid tail for LC/MS analysis. *S. aureus* wt. or mutant strains are treated with moenomycin A, which inhibits PGT activity, to accumulate Lipid II, which is then isolated by a two-step extraction procedure (see Methods). Acidic hydrolysis of the isolated Lipid II removes the undecaprenyl-phosphate chain for LC/MS analysis. (b) Chemical structure of Lipid II monophosphates after acid hydrolysis. (c) LC/MS analysis of delipidated Gly_1- and Gly_3-Lipid II monophosphates isolated from the Δ*femA* and Δ*femB* mutants, respectively (also see SI Fig. 4 and 5 for LC/MS/MS). (d) A bar graph showing ratios of crosslinking and hydrolysis activities for PBP2a, PBP2 and PBP4 with Gly_5-, Gly_3-, and Gly_1-peptidoglycan (see Methods, SI Table 1).

For Gly_5-peptidoglycan, PBP4 performed almost exclusively crosslinking, whereas 90% of products were due to hydrolysis with Gly_3-peptidoglycan. With Gly_1-peptidoglycan, PBP4 acted only as a carboxypeptidase.

To assess the relevance of the *in vitro* findings on PBP4, we quantified the percentage of hydrolyzed monomer compared to other muropeptides in cell wall isolated from Δ*femB* strains with or without *pbp4* (Figure 4a).[14a] In wildtype *S. aureus*, hydrolyzed muropeptide makes up about 10% of the total muropeptide products and this increases to 20% in the ΔfemB mutant. When *pbp4* is deleted in the Δ*femB* background, hydrolyzed muropeptide decreases to wildtype levels. Complementation of Δ*pbp4*Δ*femB* with *pbp4* doubles the amount of hydrolyzed muropeptide. We concluded that PBP4 has substantial carboxypeptidase activity in cells producing Gly_3-peptidoglycan, consistent with *in vitro* findings.

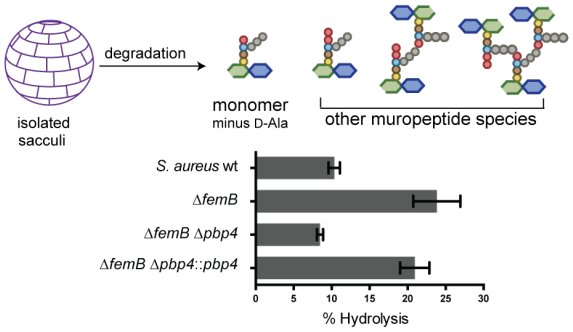
Determining whether PBP2a can crosslink Gly_3-peptidoglycan *in vivo* was more challenging than probing PBP4's role. We compared the minimum inhibitory concentrations (MIC) of three β-lactams, ceftaroline, piperacillin, and cefoxitin, against four *S. aureus* strains, wildtype, Δ*femB*, *mecA*Δ*femB*, and *mecA*Δ*femA* (Figure 4b).[15] The *mecA* strains contain a cassette that expresses PBP2a. Deleting *femB* had only a modest effect on the MICs of the three drugs. Introducing *mecA* into the Δ*femB* background, however, increased the MICs of piperacillin and cefoxitin by 8-fold and 16-fold, respectively, but did not significantly affect the MIC of ceftaroline. These findings are consistent with the *in vitro* data showing that PBP2a can crosslink Gly_3-peptidoglycan when the native PBPs are inhibited. In contrast, the *mecA*Δ*femA* strain remained susceptible to all three β-lactams, confirming the *in vitro* results that PBP2a preferentially hydrolyzes Gly_1-peptidoglycan.[16]

We have found that three *S. aureus* PBPs in MRSA show substantial differences in crosslinking versus hydrolysis behavior as the glycine branch on Lipid II is shortened. Based on our results, we draw the following conclusions. First, PBP4, although noted for its ability to make highly crosslinked peptidoglycan, primarily acts as a carboxypeptidase when there are fewer than five glycines in the branch peptide. Second, because the transpeptidase activity of *S. aureus* PBP2 is relatively insensitive to glycine branch length, this PBP must play a major role in crosslinking Gly_1-peptidoglycan to allow survival of strains lacking *femA*. Finally, PBP2a unexpectedly crosslinks Gly_3-peptidoglycan. These findings have important implications for strategies to treat MRSA infections because they imply that FemA is a better target than FemB for potentiators designed to restore susceptibility to β-lactams.[17]

ASSOCIATED CONTENT

Supporting Information.
Experimental procedures, protein purification protocol, LC/MS analysis, and Western blot analysis. This material is available free of charge via the Internet at http://pubs.acs.org

a)



b)

inhibitor (PBP selectivity)	MIC (μg/mL)			
	wt	Δ*femB*	*mecA*Δ*femB*	*mecA*Δ*femA*
ceftaroline (1, 2, 2a, 3)	2	1	2	0.25
piperacillin (1, 2, 3)	1	0.5	4	1
cefoxitin (4 > 2)	2	2	32	1

Figure 4. In cells, Gly_3-peptidoglycan undergoes hydrolysis by PBP4 yet can be crosslinked by PBP2a. (a) *S. aureus* sacculi were degraded and analyzed by LC/MS to determine the extent of D-Ala hydrolysis. The bar graph shows the percentage of hydrolyzed muropeptide compared to other depicted species. Deleting PBP4 reduces hydrolysis. (b) Minimum inhibitory concentrations (MICs) for three different β-lactams against four *S. aureus* strains, with the targeted PBPs for each β-lactam shown in parentheses. The presence of *mecA*, encoding PBP2a, in the Δ*femB* background increases the MIC of piperacillin and cefoxitin, but not ceftaroline, consistent with the *in vitro* data that PBP2a can crosslink Gly_3-peptidoglycan. In the Δ*femA* background, *mecA* does not confer resistance.

AUTHOR INFORMATION

Corresponding Authors

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

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. S. J. Kim for providing the UT34-2 and UK17 strains and Prof. A. Cheung for the MW2 Δ*pbp4*, MW2 Δ*pbp4::pbp4* strains. We also thank J. X. Wang at the Harvard Small Molecule Mass Spectrometry Facility for help with MS.

Funding Sources

This work was funded by NIH grants GM076710 (S.W.) GM066174 (D.K.) and a Singapore A*STAR NSS (Y.Q).

REFERENCES

(1) Walsh, C. T. W. T. *Antibiotics: Challenges, Mechanisms, Opportunities*; ASM press: Washington, DC, 2016.

(2) (a) Fishovitz, J.; Rojas-Altuve, A.; Otero, L. H.; Dawley, M.; Carrasco-López, C.; Chang, M.; Hermoso, J. A.; Mobashery, S. *J. Am. Chem. Soc.* **2014**, *136*, 9814; (b) Fishovitz, J.; Taghizadeh, N.; Fisher, J. F.; Chang, M.; Mobashery, S. *J. Am. Chem. Soc.* **2015**, *137*, 6500; (c) de Lencastre, H.; Oliveira, D.; Tomasz, A. *Curr. Opin. Microbiol.* **2007**, *10*, 428.

(3) Center for Disease Control and Prevention Office of Infectious Disease. https://www.cdc.gov/drugresistance/threat-report-2013 (accessed May 1, 2017).

(4) (a) Villegas-Estrada, A.; Lee, M.; Hesek, D.; Vakulenko, S. B.; Mobashery, S. *J. Am. Chem. Soc.* **2008**, *130*, 9212; (b) Lovering, A. L.; Gretes, M. C.; Safadi, S. S.; Danel, F.; de Castro, L.; Page, M. G.; Strynadka, N. C. *J. Biol. Chem.* **2012**, *287*, 32096.

(5) (a) Maidhof, H.; Reinicke, B.; Blumel, P.; Berger-Bachi, B.; Labischinski, H. *J. Bacteriol.* **1991**, *173*, 3507; (b) Henze, U.; Sidow, T.; Wecke, J.; Labischinski, H.; Berger-Bächi, B. *J. Bacteriol.* **1993**, *175*, 1612; (c) Rohrer, S.; Berger-Bächi, B. *Antimicrob. Agents Chemother.* **2003**, *47*, 837.

(6)(a) Strandén, A. M.; Ehlert, K.; Labischinski, H.; Berger-Bächi, B. *J. Bacteriol.* **1997**, *179*, 9; (b) Tschierske, M.; Ehlert, K.; Stranden, A. M.; Berger-Bachi, B. *FEMS Microbiol. Lett.* **1997**, *153*, 261.

(7)Qiao, Y.; Srisuknimit, V.; Rubino, F.; Schaefer, K.; Ruiz, N.; Walker, S.; Kahne, D. *Nat Chem Biol* **2017**, *13*, 793.

(8)(a) Ye, X.-Y.; Lo, M.-C.; Brunner, L.; Walker, D.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2001**, *123*, 3155; (b) Schwartz, B.; Markwalder, J. A.; Wang, Y. *J. Am. Chem. Soc.* **2001**, *123*, 11638; (c) VanNieuwenhze, M. S.; Mauldin, S. C.; Zia-Ebrahimi, M.; Winger, B. E.; Hornback, W. J.; Saha, S. L.; Aikins, J. A.; Blaszczak, L. C. *J. Am. Chem. Soc.* **2002**, *124*, 3656; (d) Breukink, E.; van Heusden, H. E.; Vollmerhaus, P. J.; Swiezewska, E.; Brunner, L.; Walker, S.; Heck, A. J. R.; de Kruijff, B. *J. Biol. Chem.* **2003**, *278*, 19898; (e) Lloyd, A. J.; Gilbey, A. M.; Blewett, A. M.; De Pascale, G.; El Zoeiby, A.; Levesque, R. C.; Catherwood, A. C.; Tomasz, A.; Bugg, T. D. H.; Roper, D. I.; Dowson, C. G. *J. Biol. Chem.* **2008**, *283*, 6402; (f) Huang, L.-Y.; Huang, S.-H.; Chang, Y.-C.; Cheng, W.-C.; Cheng, T.-J. R.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **2014**, *53*, 8060.

(9) (a) Lebar, M. D.; Lupoli, T. J.; Tsukamoto, H.; May, J. M.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2013**, *135*, 4632; (b) Lebar, M. D.; May, J. M.; Meeske, A. J.; Leiman, S. A.; Lupoli, T. J.; Tsukamoto, H.; Losick, R.; Rudner, D. Z.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2014**, *136*, 10874; (c) Lupoli, T. J.; Lebar, M. D.; Markovski, M.; Bernhardt, T.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2014**, *136*, 52; (d) Qiao, Y.; Lebar, M. D.; Schirner, K.; Schaefer, K.; Tsukamoto, H.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2014**, *136*, 14678.

(10) (a) Lim, D.; Strynadka, N. C. J. *Nat. Struct. Mol. Biol.* **2002**, *9*, 870; (b) Fuda, C.; Suvorov, M.; Vakulenko, S. B.; Mobashery, S. *J. Biol. Chem.* **2004**, *279*, 40802.

(11) (a) Bobba, S.; Ponnaluri, V. K. C.; Mukherji, M.; Gutheil, W. G. *Antimicrob. Agents Chemother.* **2011**, *55*, 2783; (b) Lovering, A. L.; Gretes, M. C.; Safadi, S. S.; Danel, F.; De Castro, L.; Page, M. G. P.; Strynadka, N. C. J. *J. Biol. Chem.* **2012**.

(12) (a) Davies, T. A.; Page, M. G. P.; Shang, W.; Andrew, T.; Kania, M.; Bush, K. *Antimicrob. Agents Chemother.* **2007**, *51*, 2621; (b) Moisan, H.; Pruneau, M.; Malouin, F. *J. Antimicrob. Chemother.* **2010**, *65*, 713.

(13) (a) Pinho, M. G.; Filipe, S. R.; de Lencastre, H.; Tomasz, A. *J Bacteriol* **2001**, *183*, 6525; (b) Pinho, M. G.; de Lencastre, H.; Tomasz, A. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98*, 10886.

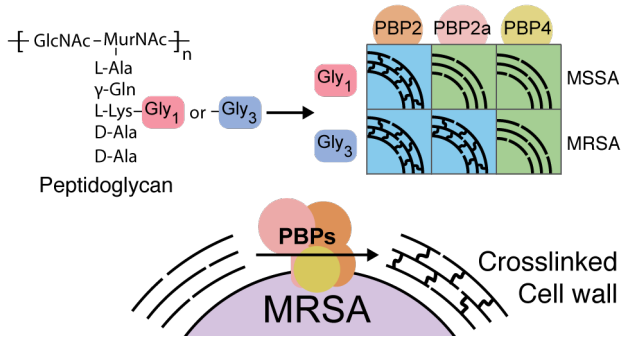
(14) (a) Memmi, G.; Filipe, S. R.; Pinho, M. G.; Fu, Z.; Cheung, A. *Antimicrob. Agents Chemother.* **2008**, *52*, 3955; (b) Chan, L. C.; Basuino, L.; Diep, B.; Hamilton, S.; Chatterjee, S. S.; Chambers, H. F. *Antimicrob. Agents Chemother.* **2015**, *59*, 2960.

(15) Sharif, S.; Kim, S. J.; Labischinski, H.; Chen, J.; Schaefer, J. *J. Bacteriol.* **2013**, *195*, 1421.

(16) As most beta-lactams do not inhibit PBP4, we suggest that the hydrolytic activity of PBP4 on Gly_1- and Gly_3-peptidoglycan may contribute to the hypersensitivity of Δ*femA* and Δ*femB* strain to beta-lactams.

(17) Fukumoto, A.; Kim, Y.-P.; Hanaki, H.; Shiomi, K.; Tomoda, H.; Ōmura, S. *J. Antibiot.* **2008**, *61*, 7.

TOC graphic


GlcNAc–MurNAc
n
L-Ala
γ-Gln
L-Lys–Gly1 or –Gly3
D-Ala
D-Ala
Peptidoglycan
PBP2
PBP2a
PBP4
Gly1
Gly3
MSSA
MRSA
PBPs
MRSA
Crosslinked
Cell wall