Supplementary Material

Impact of FtsZ Inhibition on the Localization of the Penicillin Binding Proteins in Methicillin-Resistant *Staphylococcus aureus*

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Supplemental Figures and Tables:

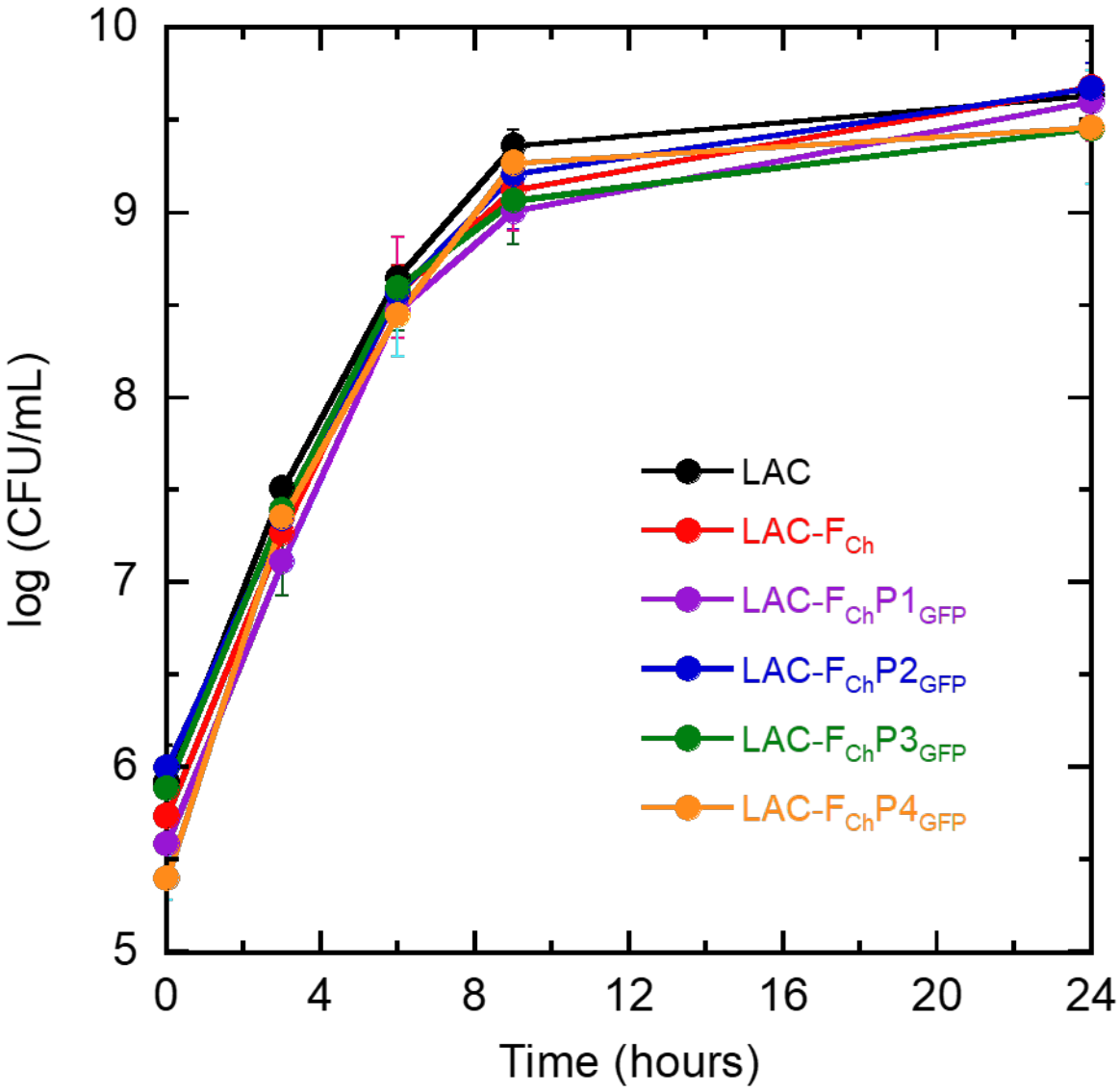

Figure S1: Growth curves associated with wild-type and genetically modified MRSA LAC strains.

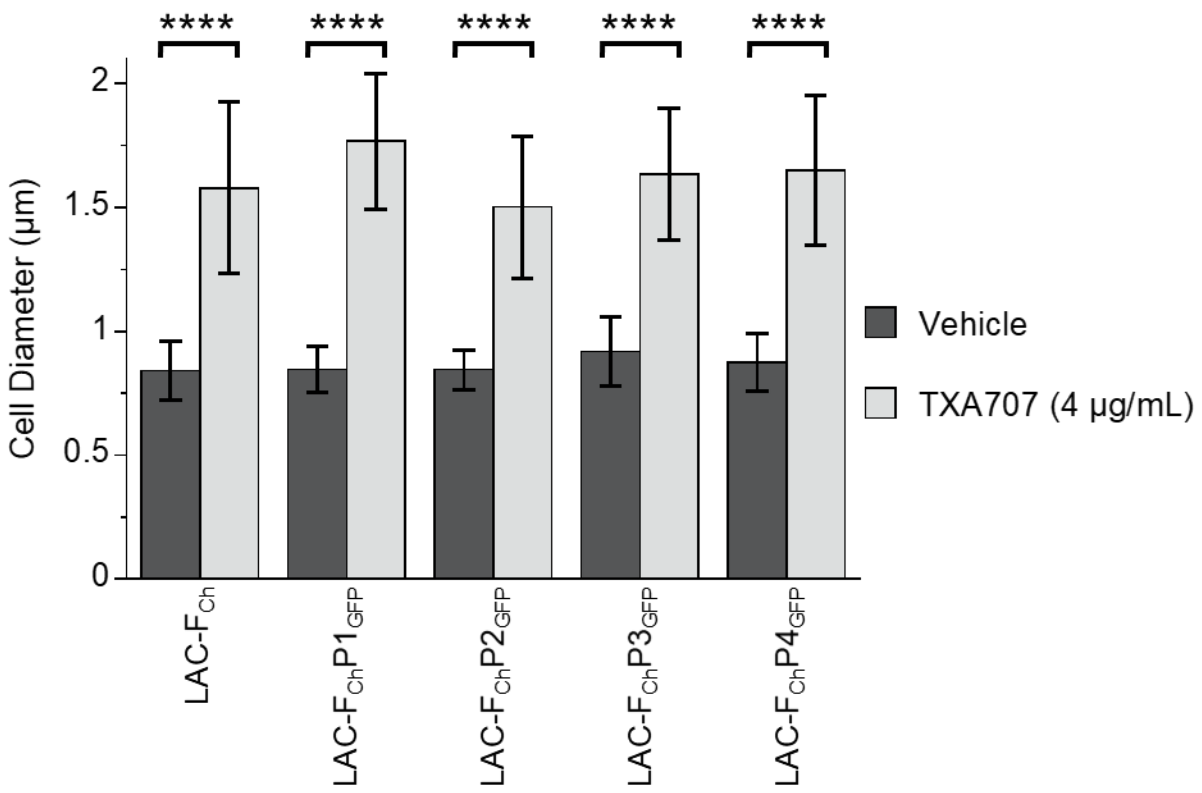

Figure S2: Impact of TXA707 treatment on the cell diameter of MRSA LAC cells expressing a FtsZ-mCherry fusion protein (LAC-F_{Ch}) or both FtsZ-mCherry and an sfGFP fusion protein of PBP1, PBP2, PBP3, or PBP4 (LAC-$F_{Ch}P1_{GFP}$, LAC-$F_{Ch}P2_{GFP}$, LAC-$F_{Ch}P3_{GFP}$, and LAC-$F_{Ch}P4_{GFP}$, respectively). Cells were treated for 3 hours with vehicle (DMSO) or 4 μg/mL (4x MIC) TXA707. The bar graph shows the average diameter of both vehicle-treated cells (n = 536, 919, 1,071, 545, and 300 for LAC-F_{Ch}, LAC-$F_{Ch}P1_{GFP}$, LAC-$F_{Ch}P2_{GFP}$, LAC-$F_{Ch}P3_{GFP}$, and LAC-$F_{Ch}P4_{GFP}$, respectively) and TXA707-treated cells (n = 315, 531, 368, 326, and 326 for LAC-F_{Ch}, LAC-$F_{Ch}P1_{GFP}$, LAC-$F_{Ch}P2_{GFP}$, LAC-$F_{Ch}P3_{GFP}$, and LAC-$F_{Ch}P4_{GFP}$, respectively). The indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in cell diameter were analyzed using a One-Way ANOVA test. **** reflects a p-value <0.0001; *** reflects a p-value in the range of $0.0001 < p < 0.001$; ** reflects a p-value in the range of $0.001 < p < 0.01$; * reflects a p-value in the range of $0.01 < p < 0.1$; n.s. denotes not significant, as reflected by a p-value >0.1.

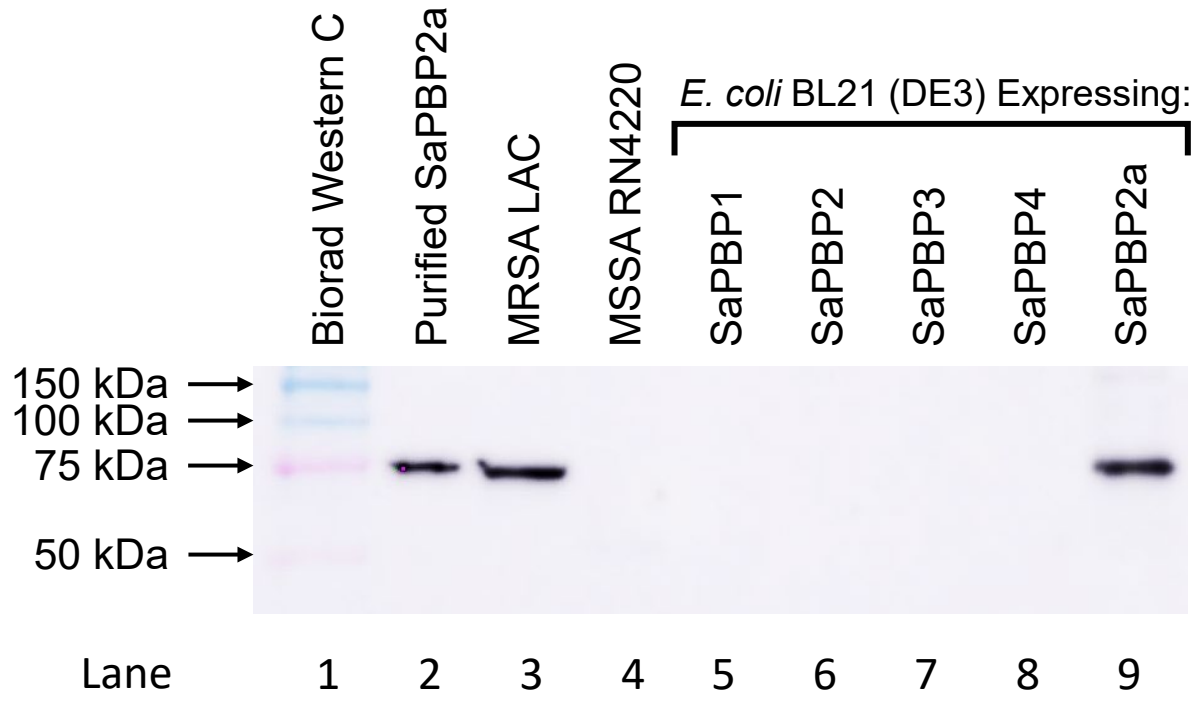

Figure S3: Western blot analysis of the mouse anti-MRSA monoclonal antibody (RayBiotech). Lane 2 contains 2.5 ng of purified *S. aureus* PBP2a (SaPBP2a), while lanes 3-9 contain lysates of MRSA LAC (lane 3), MSSA RN4220 (lane 4), and *E. coli* BL21 (DE3) cells (lanes 5-9). The *E. coli* cell lysates in lanes 5-9 are of strains expressing recombinant SaPBP1 (lanes 5), SaPBP2 (lane 6), SaPBP3 (lane 7), SaPBP4 (lane 8), or SaPBP2a (lane 9). All cell lysates contained 2.5 µg total protein.

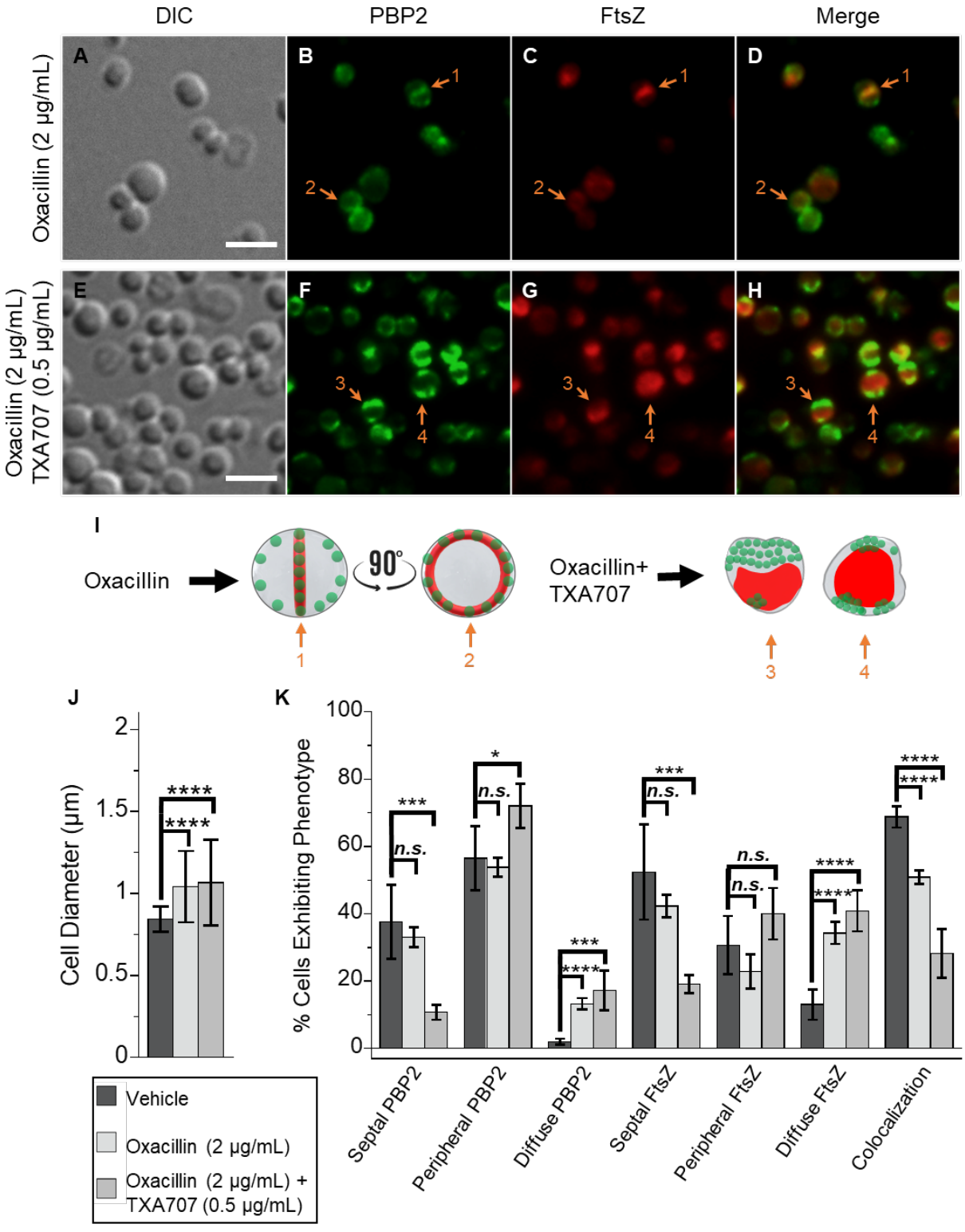

Figure S4: DIC and fluorescence micrographs of MRSA LAC-F$_{ChP2GFP}$ cells treated for 3 hours with either 2 µg/mL (1/32x MIC) oxacillin (A-D) or a combination of 2 µg/mL (1/32x MIC) oxacillin and 0.5

μg/mL (1/2x MIC) TXA707 (E-H) just prior to visualization. The localization of PBP2 (green) and FtsZ (red) is schematically depicted in panel I, with the numbered arrows in the scheme reflecting the correspondingly numbered arrows in the florescence micrographs. The scale bars for panels A-H represent 2 μm. The bar graph in panel J shows the average diameter of the vehicle-treated cells (n = 1,071), oxacillin-treated cells (n = 406), and the cells treated with a combination of both oxacillin and TXA707 (n = 432). The bar graph in panel K shows the prevalence (in %) of the various FtsZ and PBP2 phenotypes observed in the different treatment groups. Each percentage reflects an average of 5 different fields of view, with the number of cells in each field of view ranging from 61 to 247. In both panels J and K, the indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in cell diameter, FtsZ phenotype, and PBP2 phenotype were analyzed as described in the legend to Fig. S2.

TABLE S1 Activities of TXA707, various β-lactam antibiotics, and vancomycin against the wild-type and genetically modified MRSA LAC strains as well as MSSA RN4220

Strain	MIC (μg/mL)					
	TXA707	Oxacillin	Ceftriaxone	Cefotaxime	Cephalexin	Vancomycin
Wild-Type LAC	1	64	512	128	128	1
LAC-F_{Ch}	1	64	256	128	128	1
LAC-$F_{Ch}P1_{GFP}$	1	64	256	128	128	1
LAC-$F_{Ch}P2_{GFP}$	1	64	512	128	128	1
LAC-$F_{Ch}P3_{GFP}$	1	64	256	128	128	1
LAC-$F_{Ch}P4_{GFP}$	1	64	256	64	128	1
LAC-$P2A_{Ch-1}$	1	0.125	4	2	4	1
LAC-$P2A_{Ch-2}$	1	0.125	4	2	4	1
MSSA RN4220	1	0.125	4	2	4	1

TABLE S2 Plasmids used in this study

Plasmid	Description	Source
pJB38	Insertless vector for allelic exchange	J. L. Bose[a]
pJB38-sfGFP-PBP1	Construction of *sfgfp-pbpA* allele	This study
pJB38-sfGFP-PBP2	Construction of *sfgfp-pbpB* allele	This study
pJB38-sfGFP-PBP3	Construction of *sfgfp-pbpC* allele	This study
pJB38-PBP4-sfGFP	Construction of *pbpD-sfgfp* allele	This study
pJB38-mCherry-PBP2a	Construction of *mCherry-mecA* allele	This study
pJB38-PBP2a-mCherry	Construction of *mecA-mCherry* allele	This study
pCM11	Plasmid containing the gene for sfGFP	A. R. Horswill[b]
pmCherry	Plasmid containing the gene for mCherry	Takara Bio, Inc.

[a]Reference (1)

[b]Reference (2)

TABLE S3 DNA fragments (P1, P2, and P3) used in the preparation of the allelic exchange vectors for each listed strain

Strain	DNA Fragments									Resulting Plasmid
	P1			P2			P3			
	Primers[a]	Template	Description	Primers[a]	Template	Description	Primers[a]	Template	Description	
LAC-$F_{Ch}P1_{GFP}$	pJB38-pbpA up-F pbpA up-R	LAC	Upstream *pbpA*	sfGFP-pbpA-F sfGFP-pbpA-R	pCM11	sfGFP (with stop codon deleted and linker introduced)	pbpA-F pbpA-pJB38-R	LAC	*pbpA*	pJB38-sfGFP-PBP1
LAC-$F_{Ch}P2_{GFP}$	pJB38-pbpB up-F pbpB up-R	LAC	Upstream *pbpB*	sfGFP-pbpB-F sfGFP-pbpB-R	pCM11	sfGFP (with stop codon deleted and linker introduced)	pbpB-F pbpB-pJB38-R	LAC	*pbpB*	pJB38-sfGFP-PBP2
LAC-$F_{Ch}P3_{GFP}$	pJB38-pbpC up-F pbpC up-R	LAC	Upstream *pbpC*	sfGFP-pbpC-F sfGFP-pbpC-R	pCM11	sfGFP (with stop codon deleted and linker introduced)	pbpC-F pbpC-pJB38-R	LAC	*pbpC*	pJB38-sfGFP-PBP3
LAC-$F_{Ch}P4_{GFP}$	pJB38-pbpD-F pbpD-R	LAC	*pbpD* (with stop codon deleted and linker introduced)	pbpD-sfGFP-F pbpD-sfGFP-R	pCM11	sfGFP	pbpD-down-F pbpD-down-pJB38-R	LAC	Downstream *pbpD*	pJB38-PBP4-sfGFP
LAC-$P2A_{Ch\text{-}1}$	pJB38-mecA-C-F mecA-C-R	LAC	*mecA* (with stop codon deleted and linker introduced)	mecA-C-mCherry-F mecA-C-mCherry-R	pmCherry	mCherry	mecA-C-down-F mecA-C-down-F	LAC	Downstream *mecA*	pJB38-PBP2a-mCherry
LAC-$P2A_{Ch\text{-}2}$	pJB38-mecA-N-up-F mecA-N-up-R	LAC	Upstream *mecA*	mecA-N-mCherry-F mecA-N-mCherry-R	pmCherry	mCherry (with stop codon deleted and linker introduced)	mecA-N-F mecA-N-R	LAC	*mecA*	pJB38-mCherry-PBP2a

[a]The sequence of each oligonucleotide primer is listed in Table S4.

TABLE S4 Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
pJB38-pbpB up-F	CGAGGCCCTTTCGTCTTCAACGTGTATGTTGTTATACGATG
pbpB up-R	CTTTGCTCATTCATACGCGGTCCTCACTTTC
sfGFP-pbpB-F	CCGCGTATGAATGAGCAAAGGAGAAGAACTTTTC
sfGFP-pbpB-R	TTTCCGTCATAGAACCTCCTCCACCAGAACCTCCTCCACCAC TAGTGGATCCTTTGTAGAGCTC
pbpB-F	AGGAGGTTCTATGACGGAAAACAAAGGATC
pbpB-pJB38-R	CAGAGCTTGCATGCCTGCAGTTAGTTGAATATACCTGTTAAT CC
pJB38-F	CTGCAGGCATGCAAGCTCT
pJB38-R	TTGAAGACGAAAGGGCCTC
pJB38-pbpA up-F	CGAGGCCCTTTCGTCTTCAACAGGTGTTCCAAGAATATG
pbpA up-R	CTTTGCTCATTTTAATTTTTTGCTTCGCC
sfGFP-pbpA-F	CAAAAAATTAAAATGAGCAAAGGAGAAGAAC
sfGFP-pbpA-R	AGAGCCACCTCCGCCAGAACCGCCACCTCCTTTGTAGAGCT CATCCATG
pbpA-F	GTTCTGGCGGAGGTGGCTCTGCGAAGCAAAAAATTAAAATT AAAAAAAATAAAATAG
pbpA-pJB38-R	CAGAGCTTGCATGCCTGCAGTTAGTCCGACTTATCCTTG
pbpC -pJB38-up-F	CGAGGCCCTTTCGTCTTCAAATGCTGCAGTAGAAGGTAC
pbpC up-R	CTTTGCTCATAACTACCTACCTCTATTCAAAG
sfGFP-pbpC-F	GTAGGTAGTTATGAGCAAAGGAGAAGAAC
sfGFP-pbpC-R	AGAGCCACCTCCGCCAGAACCGCCACCTCCTTTGTAGAGCT CATCCATG
pbpC-F	GTTCTGGCGGAGGTGGCTCTTTAAAAAGACTAAAAGAAAAA TCAAATG
pbpC-pJB38-R	GATAAAAATAAAGACAAAGACAAATAACTGCAGGCATGCA AGCTCTG
pJB38-pbpD-F	CGAGGCCCTTTCGTCTTCAAATGAAAAATTTAATATCTATTA TCATCATTTTATG
pbpD-R	GAGGCGCCGCAGGATTTTCTTTTTCTAAATAAACGATTGATT ATC

pbpD-sfGFP-F	GAAAAAGAAAATCCTGCGGCGCCTCCATGAGCAAAGGAGAAGAAC
pbpD-sfGFP-R	TTAGTATGTTTTATTTGTAGAGCTCATCCATG
pbpD-down-F	GCTCTACAAATAAAACATACTAAAAACGGACAAG
pbpD-down-pJB38-R	CAGAGCTTGCATGCCTGCAGACAAGTAACGAAGAAGATTTTAATAG
pJB38-mecA-C-F	CGAGGCCCTTTCGTCTTCAAATGAAAAAGATAAAAATTGTTCCAC
mecA-C-R	AGAACCTCCTCCACCAGAACCTCCTCCACCACTAGTTTCATCTATATCGTATTTTTTATTACC
mecA-C-mCherry-F	ACTAGTGGTGGAGGAGGTTCTGGTGGAGGAGGTTCTATGGTGAGCAAGGGCGAG
mecA-C-mCherry-R	CACTGTTTTGCTTGTACAGCTCGTCCATGC
mecA-C-down-F	GCTGTACAAGCAAAACAGTGAAGCAATC
mecA-C-down-R	CAGAGCTTGCATGCCTGCAGGAATTTAAATGTAGAATTAAAAGGTATTAC
pJB38-mecA-N-up-F	CGAGGCCCTTTCGTCTTCAAAGCTTCTTTAGCTGTTTC
mecA-N-up-R	TGCTCACCATCAATATACTCCTTATATAAGACTACATTTG
mecA-N-mCherry-F	GAGTATATTGATGGTGAGCAAGGGCGAG
mecA-N-mCherry-R	AGAACCTCCTCCACCAGAACCTCCTCCACCACTAGTGTACAGCTCGTCCATGCC
mecA-N-F	ACTAGTGGTGGAGGAGGTTCTGGTGGAGGAGGTTCTATGAAAAAGATAAAAATTGTTCCAC
mecA-N-R	CAGAGCTTGCATGCCTGCAGTTATTCATCTATATCGTATTTTTTATTACC
pbp2a-HiFi-F	TTTAAGAAGGAGATATACATATGTCAAAAGATAAAGAAATTAATAATACTATTGATG
pbp2a-HiFi-R	TCAGTGGTGGTGGTGGTGGTGTTCATCTATATCGTATTTTTTATTACCG

Supplemental Methods:

Cloning, expression, and purification of *S. aureus* PBP2a (SaPBP2a). The genomic DNA of MRSA LAC was extracted from an overnight culture using the DNeasy UltraClean Microbial Kit (Qiagen). The *mec*A gene was then PCR-amplified from the genomic DNA using Q5 High-Fidelity DNA polymerase (New England Biolabs) and the primers pbp2a-HiFi-F and pbp2a-HiFi-R (the sequences of which are listed in Table S4). These primers were designed to remove the N-terminal transmembrane domain and introduce a 6x His-tag at the *C*-terminus of the recombinant PBP2a protein. The expression vector pET-22b(+) (Novagen-EMD Chemicals) was linearized using the restriction enzymes *Nde*I and *Xho*I. The linearized plasmid and the amplified *mec*A gene were then combined, assembled, and circularized using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs), with the resulting recombinant plasmid being used to transform *E. coli* NEB5-α cells. The transformed *E. coli* NEB5-α cells were grown on Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin. Single colonies were selected for colony PCR to verify the presence of the proper insert in the pET-22b(+) plasmid. The recombinant plasmid was then isolated from *E. coli* NEB5-α and its sequence subsequently verified. This recombinant plasmid was then transformed into *E. coli* BL21 (DE3) cells, and the transformed cells were plated on LB agar containing 100 μg/mL ampicillin.

A single colony of the transformed *E. coli* BL21 (DE3) cells was then isolated and grown overnight at 37 °C in 20 mL of ampicillin-containing LB broth. The overnight culture was diluted into 4 L of autoinduction terrific broth (3), followed by incubation at 37 °C for 6 hours. The cultures were then incubated for an additional 48 hours at 25 °C. The cells were then harvested by centrifugation at 5,000 x g for 15 minutes at 4 °C. The cell pellets were then resuspended in 50 mL of 10 mM sodium phosphate (pH 7.6), 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride

(PMSF), and 10% (vol/vol) glycerol and stored at -80 °C. Cells were lysed by ultrasonication for 15 minutes at 0 °C using a Qsonica Q500 sonicator equipped with a 1/2-inch probe, with an on/off cycle of 10 seconds and an amplitude set at 60%. The lysate was centrifuged at 10,000 x g for 30 minutes at 4 °C. The resulting supernatant was added to 5 mL of Talon metal affinity resin (Clontech Laboratories, Inc.) and shaken for 20 minutes at 4 °C. The resin was then washed with 50 mL of buffer containing 10 mM sodium phosphate (pH 7.6), 250 mM NaCl, and 10% (vol/vol) glycerol (buffer A) and packed into a gravity flow column. The column was washed with 10 mL of buffer A, followed by 25 mL of buffer containing 10 mM sodium phosphate (pH 7.6), 10 mM imidazole, and 250 mM NaCl (buffer B). The protein was then eluted using 15 mL of elution buffer containing 10 mM sodium phosphate (pH 7.6), 150 mM imidazole, and 250 mM NaCl (buffer C), and 500 μL fractions were collected. Each fraction was analyzed by SDS-PAGE and fractions containing protein were combined. The combined fractions were dialyzed overnight against 4 L of buffer containing 100 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 10% (vol/vol) glycerol. The resulting dialysate was concentrated to a volume of 1 mL using Amicon Ultra 10K filters (EMD Millipore, Inc.). The final protein concentration was quantified using a Pierce BCA Protein Assay Kit (ThermoFisher).

Supplemental References

1. Bose JL, Fey PD, Bayles KW. 2013. Genetic tools to enhance the study of gene function and regulation in *Staphylococcus aureus*. Appl Environ Microbiol 79:2218-24.
2. Lauderdale KJ, Malone CL, Boles BR, Morcuende J, Horswill AR. 2010. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. J Orthop Res 28:55-61.
3. Grabski A, Mehler M, Drott D. 2005. The Overnight Express Autoinduction System: High-density cell growth and protein expression while you sleep. Nat Methods 2:233-235.