INSIGHTS INTO FTSZ INHIBITORS AND THEIR USE AGAINST ANTIBIOTIC-RESISTANT BACTERIAL PATHOGENS

by

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Written under the direction of
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ABSTRACT OF THE DISSERTATION

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Antibiotic resistance is one of the biggest public health challenges of our time. The emergence and spread of drug-resistant pathogens threatens our ability to treat common infections. This problem is exacerbated by the dwindling pipeline of new antimicrobial agents. This dissertation explores the mechanism by which FtsZ inhibitors overcome the resistance properties of methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA resistance to β-lactam antibiotics is predominantly driven by the acquisition of a fifth penicillin binding protein (PBP), termed PBP2a. β-lactam antibiotics have a low binding affinity for PBP2a, allowing MRSA to survive in the presence of these drugs. When β-lactam antibiotics are used in combination with a FtsZ inhibitor they become active against MRSA. To probe the mechanism underlying this behavior, we first determined the relative binding affinities of various β-lactam antibiotics for *S. aureus* PBP1, PBP2, PBP3, and PBP4. Using this information, we explored the relationship between PBP targeting selectivity and the extent of antibacterial synergy observed when combining different β-lactam antibiotics with a FtsZ inhibitor. Our findings indicate that β-lactam antibiotics with a high binding affinity for PBP2 exhibit the greatest extent of synergistic activity with FtsZ inhibitors.
To further understand the nature of this synergistic relationship, we analyzed the impact of FtsZ inhibition on the relative localization of FtsZ and the five PBPs present in MRSA using fluorescence microscopy. Our results suggest that PBP2 plays a major role in the cell wall remodeling and repair necessitated by FtsZ inhibition and that PBP2a does not complement this function. Without a complementary contribution from PBP2a, PBP2 becomes highly vulnerable to PBP2-selective β-lactam antibiotics, thereby underscoring the high degree of synergistic activity between these drugs and FtsZ inhibitors. In addition to these findings, the development and validation of a fluorescent FtsZ inhibitor (BOFP) is also presented. Our results show that BOFP can be utilized to visualize and study FtsZ in a broad range of both Gram-negative and Gram-positive bacterial pathogens of acute clinical importance. This powerful tool will enable us to identify new broad-spectrum FtsZ inhibitors and understand their mechanisms of action, while expedite the knowledge on FtsZ.
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INTRODUCTION

Antibiotic resistance is a burgeoning problem that affects public health worldwide. It is estimated that by the year 2050 antibiotic-resistant infections could claim 10 million lives each year and impose a major burden on the global economy (1). The rapid emergence of antibiotic-resistant microorganisms has endangered the efficacy of current arsenal of antibiotics (2). In the United States of North America, the Centers for Disease Control and Prevention (CDC) states that approximately 23,000 patients die each year due to infections caused by multidrug resistant microorganisms (3). Although resistance is a natural mechanism of microbial adaptation in their environment, the overuse and misuse of antibiotics over the years has enriched the population of clinical pathogens with resistant phenotypes (4-7). In addition, the development and manufacturing of new antibiotics has slumped to an all-time low (8, 9). This situation places us in a very vulnerable scenario that if not address properly could eventually advance society towards a post-antibiotic era (10).

In response to this crisis, bacterial cell division has emerged as a new attractive antibiotic targeting pathway to counteract multidrug-resistant pathogens (2, 11-16). Filamenting temperature-sensitive mutant Z (FtsZ), a highly conserved bacterial cytoskeletal protein is a key modulator of cell division in most bacterial species (17). The assembly of FtsZ at mid-cell establishes a cytoskeletal structure known as the Z-ring. Upon formation of the Z-ring, the cell division machinery (divisome), gets recruited bacterial cell division initiates (17-22). The structural Z-ring facilitates bacterial cytokinesis by treadmilling around the site of assembly (2, 20, 23-25). The force generated throughout the treadmilling of the Z-ring, empowers the Penicillin Binding Protein’s (PBP’s) associated with the divisome to elegantly synthesized a brand-new peptidoglycan cell wall (20, 25-27). Previous studies had demonstrated that targeting FtsZ by
either perturbing the function and dynamics of the cell division machinery or affecting the structural integrity of FtsZ, resulted in an impressive antimicrobial activity property against methicillin-resistant *Staphylococcus aureus* (MRSA) and other bacterial pathogens (13, 14, 28-35). Additionally, it has been demonstrated that inhibition of FtsZ in MRSA causes it to become susceptible to β-lactam antibiotics (36-42).

β-lactams are a class of antibiotics target the transpeptidase domain of the PBPs, by inactivating the catalytic domain in charge of cross-linking the bacterial cell wall (43-47). Their discovery date backs 1928, when Alexander Fleming accidentally discovered penicillin. This finding revolutionized medicine allowing physicians to treat life-threatening illnesses such as bacterial endocarditis, meningitis, pneumococcal pneumonia, gonorrhea, and syphilis with little to no side effects (48). Resistance to β-lactam antibiotics in MRSA derived from the acquisition of two important elements. At first *S. aureus* develop resistance to penicillin by the incorporating a penicillinase into its genome(49-51). Methicillin was then introduced as a penicillinase resistant β-lactam antibiotic, it became very successful for the treatment of *S. aureus* infections during the 1960s. However, it was not long for *S. aureus* to develop resistance to methicillin. This second event of resistance originated from the acquisition of mobile genetic element containing the gene known as *mecA*. This gene encodes for an additional PBP that possesses a low binding affinity against most β-lactam antibiotics, commonly referred to as PBP2a(52-56).

The overall goal of this work is to understand the molecular basis underlying the mechanism of synergy associated between a FtsZ inhibitors and β-lactam antibiotics. Particularly understanding what drives the resensitization of MRSA to β-lactam antibiotics, and how can these findings propel pharmacological strategies that could be used to increase the lifespan of FtsZ inhibitors against MRSA. The work presented here, provides evidence on the direct functionality
of PBP2a during cell division under the pressure of β-lactam antibiotics, and elucidating a mechanistic model for how MRSA is sensitized once again to β-lactam antibiotics with the help of these FtsZ inhibitors. In addition, we present the development of a fluorescent FtsZ inhibitor (BOFP) that permits the direct visualization of FtsZ without the need of extensive bacterial genetics against both Gram-negative and Gram-positive bacterial pathogens. With the end of facilitating the development of a broad spectrum FtsZ inhibitor capable of targeting both Gram-negative and Gram-positive bacterial pathogens and enrich our understanding of FtsZ mediated bacterial cell division.
CHAPTER I

β-LACTAM ANTIBIOTICS WITH A HIGH AFFINITY FOR PBP2 ACT SYNERGISTICALLY WITH THE FTSZ-TARGETING AGENT TXA707 AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS


ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA) is a multidrug-resistant pathogen that poses a significant risk to global health today. We have developed a promising new FtsZ-targeting agent (TXA707) with potent activity against MRSA isolates resistant to current standard-of-care antibiotics. We present here results that demonstrate differing extents of synergy between TXA707 and a broad range of β-lactam antibiotics (including six cephalosporins, two penicillins, and two carbapenems) against MRSA. To explore whether there is a correlation between the extent of synergy and the preferential antibacterial target of each β-lactam, we determined the binding affinities of the β-lactam antibiotics for each of the four native penicillin-binding proteins (PBPs) of S. aureus using a fluorescence anisotropy competition assay. A comparison of the resulting PBP binding affinities with our corresponding synergy results reveals that β-lactams with a high affinity for PBP2 afford the greatest degree of synergy with TXA707 against MRSA. In addition, we present fluorescence and electron microscopy studies that suggest a potential mechanism underlying the synergy between TXA707 and the β-lactam antibiotics. In this connection, our microscopy results show a disruption of septum formation in TXA707-treated MRSA cells, with a concomitant mislocalization of the PBPs from midcell to nonproductive peripheral sites. Viewed as a whole, our results indicate that PBP2-targeting β-lactam antibiotics are optimal synergistic partners with FtsZ-targeting agents for use in combination therapy of MRSA infections.
INTRODUCTION

The emergence of multidrug-resistant (MDR) bacterial pathogens has become a global threat to public health (3, 10). Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) is an MDR bacterial pathogen associated with a high mortality rate in both hospital and community settings (50, 57, 58). In particular, MRSA is resistant to most \(\beta\)-lactam antibiotics, including penicillins, cephalosporins, and carbapenems (51). The antibacterial targets of \(\beta\)-lactam antibiotics are the penicillin-binding proteins (PBPs), with \textit{S. aureus} expressing four native PBPs (PBP1, PBP2, PBP3, and PBP4) (59, 60). In addition to these four PBPs, MRSA also expresses a fifth PBP (PBP2A) which, unlike the native \textit{S. aureus} PBPs, is a poor target for the \(\beta\)-lactams (54, 56).

The PBPs are a family of proteins that are involved in the final steps of bacterial cell wall assembly, particularly at the septa of dividing cells (46, 47). In \textit{S. aureus}, all four native PBPs are known to localize to the septa of dividing cells, with each possessing a catalytic transpeptidase (TPase) activity that is important for cross-linking peptidoglycan strands in the newly forming cell wall (61-65). PBP2 is unique among the PBPs in that it has two distinct catalytic domains, making it the only bifunctional PBP in \textit{S. aureus} (55, 59, 66). One catalytic domain of PBP2 is responsible for its TPase activity, while the other catalytic domain has transglycosylation (TGase) activity, which catalyzes the cross-linking of glycan moieties to form peptidoglycan strands (66). Pinho and coworkers have demonstrated that the ability of MRSA to survive in the presence of \(\beta\)-lactam antibiotics requires cooperative functioning between PBP2 and PBP2A (52, 55, 67).

The function of the PBPs in \textit{S. aureus} are controlled both spatially and temporally throughout the cell cycle (36). A key protein involved in this spatial and temporal regulation is FtsZ, which ensures the proper localization of the PBPs to the septum during division (21, 24, 45, 68). We have previously shown that the FtsZ-targeting agent \textbf{TXA707} (see structure in Fig. 1.S1
in the appendix A) acts synergistically with the third-generation cephalosporin **cefidinir** against MRSA (38). We sought to determine the extent to which other β-lactam antibiotics synergize with **TXA707** against MRSA and how the synergy is impacted by the PBP binding affinity and selectivity of the β-lactams. Although the PBPs have long been established as the targets of β-lactam antibiotics, the binding affinities and selectivities of these drugs for the individual PBPs of *S. aureus* have not been well characterized. Here, we determined the binding affinities of 10 representative β-lactam antibiotics (including six cephalosporins, two penicillins, and two carbapenems) for the four native PBPs of *S. aureus*. We then determined how the PBP binding selectivities of the β-lactams correlate with the extent to which the drugs synergize with **TXA707** against MRSA. Our results indicate that β-lactam antibiotics with a high affinity for PBP2 afford the greatest degree of synergy with **TXA707**.
MATERIALS AND METHODS

Compounds, antibiotics, and MRSA COL. TXA707 was synthesized as previously described (41). Cefdinir, ceftriaxone (sodium salt), cefotaxime (sodium salt), cefepime HCl, and cefradine were obtained from TOKU-E (Bellingham, WA). Oxacillin (sodium salt), ticarcillin (disodium salt), and cephalexin were obtained from Sigma-Aldrich (St. Louis, MO). Imipenem and ertapenem were obtained from LKT Laboratories (St. Paul, MN) and Ontario Chemicals (Guelph, Ontario, Canada), respectively. Bocillin was obtained from Thermo Fisher Scientific. MRSA COL was provided by Alexander Tomasz (Rockefeller University, New York, NY).

Cloning, expression, and purification of the S. aureus PBPs. The pbpA, pbpB, pbpC, and pbpD genes that encode S. aureus PBP1, PBP2, PBP3, and PBP4 were amplified by PCR using genomic DNA extracted from MRSA COL. The primers used for these amplifications are listed in Table 1.S1 in the supplemental material. For PBP1, PBP2, and PBP3, the primers were designed to remove the N-terminal transmembrane domain. For PBP4, the primers were designed to remove both the N-terminal signal peptide sequence and the C-terminal transmembrane domain. Each amplified gene was cloned into the pET-22b(+) plasmid (Novagen-EMD Chemicals, Inc.) using the NEBuilder HiFi DNA assembly cloning kit (New England BioLabs, Inc.), such that a 6× His tag was introduced at the C terminus of each recombinant gene product. The sequences of all recombinant plasmids were verified by sequence analyses, and the plasmids were then transformed into Escherichia coli BL21(DE3) cells.

Transformed E. coli were grown on Luria-Bertani (LB) agar plates containing 100 µg/mL of ampicillin. Single colonies were isolated and grown in 20 ml of ampicillin-containing LB broth overnight at 37 °C. The overnight cultures were diluted into 4 L of autoinduction terrific broth (69), followed by incubation at 37 °C for 6 hours. The cultures were then incubated for an
additional 24 to 32 hours at 30 °C. The cells were harvested by centrifugation at 5,000 × 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, with an on/off cycle of 10 seconds at 60 W. The lysates centrifuged at 10,000 × g for 30 minutes at 4 °C. The resulting supernatants were 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 by 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. For PBP1, PBP2, and PBP3, 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 dialysates were concentrated to 1 mL of volume using Amicon Ultra 10K filters (EMD Millipore, Inc.). The combined elution fractions of PBP4 were dialyzed overnight against 4 L of buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 5% (vol/vol) glycerol and then concentrated to 2 mL. Protein concentrations were quantified by using a bicinchoninic acid assay, with final protein concentrations ranging from 13 to 26 mg/mL.
Fluorescence anisotropy assays for Bocillin binding to the PBPs. Fluorescence anisotropy experiments were performed using an Aviv model ATF105 spectrofluorometer (Aviv Biomedical, Lakewood, NJ). In these experiments, bandwidths were set to 4 nm in both the excitation and the emission directions, with the excitation and emission wavelengths being set at 488 and 510 nm, respectively. For PBP1, PBP2, and PBP3, Bocillin (1 µM) was titrated with increasing concentrations (ranging from 0 to 2.5 µM) of protein in 150 µL of buffer containing 100 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 5% (vol/vol) glycerol. After each protein addition, the samples were equilibrated for 5 minutes at 25 °C, whereupon the fluorescence anisotropy was measured at 25 °C. For PBP4, individual samples containing 1 µM Bocillin and a protein concentration ranging from 2 to 50 µM were prepared in 150 µL of buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 5% (vol/vol) of glycerol. The fluorescence anisotropy of each sample was then measured at 25 °C every 6 seconds for 15 minutes. A quartz ultra-micro cell (Hellma, Inc.) with a 2- by 5-mm aperture and a 15-mm center height was used for all measurements. The path lengths in the excitation and emissions directions were 1 and 0.2 cm, respectively. All steady-state anisotropy experiments were conducted in triplicate, with the reported anisotropies reflecting the average values.

Competition fluorescence anisotropy assays. For PBP1, PBP2, and PBP3, individual samples containing 1 µM Bocillin and β-lactam antibiotic at a concentration ranging from 0 to 7.5 mM were prepared in 150 µL of buffer containing 100 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 5% (vol/vol) of glycerol. The reactions were then initiated by addition of 2 µM protein. After 5 minutes of equilibration at 25°C, the fluorescence anisotropy was then recorded as described above. For PBP4, individual samples containing 1 µM Bocillin and β-lactam antibiotic at a concentration ranging from 0 to 15 mM were prepared in 150 µL of buffer containing 20 mM Tris-
HCl (pH 7.4), 500 mM NaCl, and 5% (vol/vol) of glycerol. The reaction was then initiated by the addition of 50 μM protein. Time-dependent fluorescence anisotropy was then acquired as described above.

**MIC assay.** MIC assay was conducted in accordance with Clinical and Laboratory Standards Institute guidelines for broth microdilution(70). Briefly, log-phase MRSA COL was added to 96-well microtiter plates (at 5 × 10^5 CFU/mL) containing 2-fold serial dilutions of compound or β-lactam antibiotic in cation-adjusted Mueller-Hinton (CAMH) broth, with each compound concentration being present in duplicate. The final volume in each well was 0.1 mL, and the microtiter plates were incubated aerobically for 24 hours at 37 °C. Bacterial growth was monitored by measuring the optical density at 600 nm using a VersaMax plate reader (Molecular Devices, Inc.), with the MIC being defined as the lowest compound concentration at which growth was ≥90% inhibited.

**Time-kill assay for synergy.** Exponentially growing MRSA COL was diluted in CAMH broth to a final count of 10^5 CFU/mL. The colony count at time zero was verified by plating serial dilutions of the culture in duplicate on tryptic soy agar (TSA) plates. The initial culture was aliquoted into tubes containing either 0.5× MIC of TXA707, 0.008× MIC of the β-lactam antibiotic, or both agents combined. An equivalent volume of dimethyl sulfoxide (DMSO) was added to the vehicle control tube. The cultures were then incubated at 37 °C with shaking. The CFU/mL in each culture was determined over time by withdrawing samples at time points ranging from 3 to 24 hours and plating appropriate serial dilutions on to TSA plates. All TSA plates were incubated at 37 °C, and the CFU/mL at each time point determined by counting colonies after 24 hours.
**Transmission electron microscopy.** Log-phase MRSA COL cells were diluted to an optical density at 600 nm of 0.1 and then cultured at 37 °C for 3 hours in the presence of DMSO vehicle, TXA707 alone at a concentration of 16 μg/mL (8× MIC), or a combination of TXA707 at a concentration of 2 μg/mL (1× MIC) and either cefidinir at a concentration of 2 μg/mL (0.008× MIC) or ceftriaxone at a concentration of 16 μg/mL (0.008× MIC). The cultures were then centrifuged at 16,000 × g for 3 minutes at room temperature. The resulting bacterial pellets were washed with 1 mL of phosphate-buffered saline (PBS) and resuspended in 500 μL of 100 mM cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde and 4% paraformaldehyde. The fixed bacterial cells were then postfixed in buffered osmium tetroxide (1%), subsequently dehydrated in a graded series of ethanol, and embedded in epon resin. Thin sections (90 nm) were cut on a Leica EM UC6 ultramicrotome. Sectioned grids were then stained with a saturated solution of uranyl acetate and lead citrate. Images were captured with an AMT XR111 digital camera at 80 kV on a Philips CM12 transmission electron microscope.

**Fluorescence microscopy.** Log-phase MRSA COL cells were diluted to an optical density at 600 nm of 0.1 and then cultured at 37 °C for 3 hours in the presence of DMSO vehicle or TXA707 at a concentration of 16 μg/mL (8× MIC). The cultures were then centrifuged at 16,000 × g for 3 minutes at room temperature. The resulting bacterial pellets were washed with 1 mL of PBS, resuspended in 100 μL of PBS containing 2 μM Bocillin, and incubated in the dark for 10 minutes at room temperature. The cells were then washed with 1 mL of PBS and resuspended in 500 μL of PBS. Each cell suspension (2.5 μL) was placed on a thin layer of 1.5% agarose in PBS mounted on a microscope slide. Differential interference contrast and fluorescence visualization of the cells were performed using an Olympus BX50 microscope equipped with an X-cite Exacte 200W mercury lamp and a 100× Olympus UPLSAPO oil immersion objective (1.40 aperture). Images
were captured using a QImaging Retiga R3 charge-coupled device camera and processed with the Ocular version 1 software package (QImaging).
RESULTS

Intrinsic activities of TXA707 and the β-lactam antibiotics against MRSA COL. We investigated 10 different β-lactam antibiotics, including 6 cephalosporins (cefradine, cephalexin, cefdinir, cefotaxime, ceftriaxone, and cefepime), 2 penicillins (oxacillin and ticarcillin), and 2 carbapenems (ertapenem and imipenem), for synergy with TXA707 against MRSA COL. As an initial step in these investigations, we determined the intrinsic activities of the 10 β-lactams and TXA707 against this bacterial strain, with the resulting MIC values being listed in Table 1.1. TXA707 exhibits potent activity against MRSA COL (MIC = 2 μg/mL). In contrast, all the β-lactams studied are associated with poor activity, with MICs ranging from 32 to 2,048 μg/mL.

Binding affinities of the β-lactam antibiotics for the PBPs of S. aureus. We sought to determine the binding affinities of the 10 β-lactam antibiotics for PBP1, PBP2, PBP3, and PBP4 of S. aureus using a competition fluorescence anisotropy assay and Bocillin (Thermo Fisher Scientific, Waltham, MA) as the fluorescent ligand. Before conducting these competition experiments, we first characterized the binding of Bocillin itself to each of the PBPs. To this end, we monitored the impact of increasing concentrations of PBP1, PBP2, or PBP3 on the fluorescence anisotropy of 1 μM Bocillin, with the resulting anisotropy titration curves being shown in Fig. 1.1. Note that the addition of each protein increases the fluorescence anisotropy of Bocillin, indicative of ligand binding to each host protein. Furthermore, each anisotropy titration curve is associated with an inflection point at approximately 1 μM protein, consistent with Bocillin binding to each target protein with a 1:1 stoichiometry. The anisotropy titration results also reveal that 1 μM Bocillin is fully saturated by a 2 μM concentration of each PBP. These results suggest that Bocillin targets S. aureus PBP1, PBP2, and PBP3 with a similar affinity.
S. aureus PBP4 has been previously reported to exhibit β-lactamase activity (71). Our fluorescence anisotropy studies with PBP4 and Bocillin are consistent with this observation. Specifically, we find that the fluorescence anisotropy of Bocillin decreases as a function of time in the presence of PBP4 (Fig. 1.2A). This observation suggests that Bocillin is being hydrolyzed by PBP4, with the hydrolysis product being subsequently released from the protein binding pocket. We therefore monitored the time-dependent fluorescence anisotropy of 1 μM Bocillin as a function of PBP4 concentration, with representative time-dependent profiles at PBP4 concentrations of 2, 5, 10, 15, and 50 μM being shown in Fig. 1.2A. A plot of initial fluorescence anisotropy versus PBP4 concentration reveals that 50 μM PBP4 is required to fully complex with 1 μM Bocillin (Fig. 1.2B), a protein concentration 25-fold higher than that indicated above for the full saturation of 1 μM Bocillin by PBP1, PBP2, or PBP3. These results suggest that Bocillin binds PBP4 with a lower affinity than the other three PBPs.

Armed with the PBP concentrations required to fully complex 1 μM Bocillin, we then proceeded to investigate the binding of the 10 β-lactam antibiotics noted above to PBP1, PBP2, PBP3, and PBP4 by monitoring the fluorescence anisotropy of 1 μM Bocillin in the presence of a saturative PBP concentration and increasing concentrations of β-lactam. In this competition anisotropy assay, β-lactam binding to PBP1, PBP2, or PBP3 is correlated with a proportional decrease in the observed anisotropy of Bocillin. With regard to PBP4, β-lactam binding is correlated with a proportional decrease in the initial velocity of Bocillin hydrolysis, as determined from time-dependent anisotropy profiles like those shown in Fig. 1.2A. Representative competition anisotropy curves for each of the four native PBPs are shown in Fig. 1.3. These representative anisotropy curves reflect the competition results for 6 of the 10 β-lactams examined,
with the corresponding competition results for the other 4 β-lactams being shown in Fig. 1.S2 in the supplemental material.

The binding of β-lactams to the PBPs is associated with an irreversible (covalent) acylation interaction that precludes determination of binding affinity in the form of an equilibrium $K_d$ value. Instead, we assessed binding affinity in the form of an inhibitory concentration reflecting the concentration of β-lactam required to reduce the observed anisotropy of Bocillin by 50% (IC$_{50}$). To this end, we analyzed the competition anisotropy curves with the following relationship:

$$r_{obs} = r_f + \frac{(r_b-r_f)}{1+10^{(\log IC_{50}-C)-n_H}} \quad \text{(Eq. 1.1)}$$

In this relationship, $C$ is the concentration of β-lactam, $r_{obs}$ is the observed fluorescence anisotropy of Bocillin, $n_H$ is the Hill slope, and $r_f$ and $r_b$ are the fluorescence anisotropies of free and PBP-bound Bocillin, respectively.

The IC$_{50}$ values resulting from our analyses described above are summarized in Table 1.2, with lower IC$_{50}$s being indicative of higher PBP binding affinities. Inspection of these data provides information with regard to the PBP binding selectivities of the β-lactams, with a difference in IC$_{50}$ of ≥3-fold being viewed as reflecting selective binding. In this connection, cefradine and cephalaxin are selective for PBP3, whereas ceftriaxone, cefotaxime, ertapenem, and cefepime are selective for PBP2. Cefdinir, oxacillin, and ticarcillin selectively target both PBP2 and PBP3, while imipenem selectively targets PBP1, PBP2, and PBP3. None of the β-lactams examined target PBP4 with a high degree of affinity or selectivity.

**Synergistic activities of the β-lactam antibiotics with TXA707 against MRSA COL.** We used a time-kill approach to assess the relative potential of the 10 β-lactams to act synergistically with
against MRSA COL. To this end, we monitored bactericidal activity in the presence of a sub-MIC concentration of TXA707 alone (at 0.5× MIC), β-lactam alone (at 0.008× MIC), or a combination of both. Figure 1.4 shows the resulting kill curves for the same six β-lactam antibiotics represented in the competition anisotropy curves depicted in Fig. 1.3, with the corresponding kill curves for the other four β-lactams examined being shown in Fig. 1.5 in the supplemental material. No killing is evident in the presence of any of the β-lactams alone, with growth comparable to that associated with vehicle being observed instead. Similarly, growth is also observed in the presence of TXA707 alone, albeit less than that associated with vehicle or β-lactam alone. In striking contrast to TXA707 or β-lactam alone, the combination of TXA707 with each of the β-lactams is bactericidal. Thus, all 10 β-lactams exhibit bactericidal synergy with TXA707. However, the magnitude of kill, and therefore synergy, varies depending on the β-lactam. Figure 1.5 highlights this variability by plotting the calculated difference between the log(CFU/mL) at 24 relative to 0 hours [log (CFU/mL)_{24} − log(CFU/mL)_{0}] for each agent alone and in combination. Inspection of these data reveals that cefdinir, imipenem, ceftriaxone, cefotaxime, ertapenem, ticarcillin, and oxacillin result in >4 logs of kill when combined with TXA707. The combination of cefepime and TXA707 results in a less robust degree of kill (∼3 logs), with the combination of cefradine or cephalaxin and TXA707 yielding the lowest degree of kill (∼2 logs).

Impact of TXA707 alone or in combination with select β-lactam antibiotics on septum formation in MRSA COL. We used transmission electron microscopy (TEM) to compare the morphology of MRSA COL cells treated with vehicle relative to those treated with TXA707 at 8× MIC for 3 hours. As expected, vehicle-treated cells are able to form septa at midcell and undergo division (Fig. 1.6A). In contrast, TXA707-treated cells become enlarged, increasing in average
diameter from 0.8 to 1.6 μm. In addition, no midcell septa are evident in TXA707-treated cells, with pronounced invaginations of the cell wall being induced at distinct sites on the cell periphery (Fig. 1.6B). MRSA COL cells treated for 3 hours with a combination of TXA707 at only 1× MIC (an 8-fold lower concentration than that used for treatment with the compound alone) and either cefdinir or ceftriaxone at 0.008× MIC exhibit a similar morphological behavior, with the induced cell wall invaginations being even larger and more pronounced (Fig. 1.6C and D).

**Impact of TXA707 on the septal localization of the PBPs in MRSA COL.** We next used fluorescence microscopy to monitor the impact of TXA707 treatment (at 8× MIC for 3 hours) on localization of the PBPs in MRSA COL. To this end, we labeled MRSA COL cells with 2 μM Bocillin and compared the fluorescence pattern associated with vehicle- versus TXA707-treated cells. Recall that at 1 μM, Bocillin binds stoichiometrically to PBP1, PBP2, and PBP3, with its affinity for these PBPs being significantly higher than its affinity for PBP4. Thus, it is likely that Bocillin is labeling PBP1, PBP2, and PBP3 in the MRSA cells. In cells treated with vehicle alone, the majority of the Bocillin fluorescence is localized to the septum that is able to form at midcell (Fig. 1.7A and B). This observation is consistent with the localization of PBP1, PBP2, and PBP3 to the septa of dividing cells. In contrast, in TXA707-treated cells, no septal Bocillin fluorescence is evident at midcell. Instead, the Bocillin fluorescence is localized to distinct regions of the cell periphery (Fig. 7C and D), a behavior suggesting that the TXA707-induced invaginations of the cell wall observed in the TEM characterizations may reflect mislocalized PBPs and possibly other septal components (compare Fig. 1.6B and 1.7D). MRSA COL cells treated with a combination of TXA707 and β-lactam could not be sufficiently labeled with Bocillin, as the nonfluorescent β-lactam competes with the Bocillin fluorophore, thereby reducing or eliminating the observed fluorescence. In the aggregate, our microscopy results suggest that TXA707 treatment disrupts
septum formation by causing the mislocalization of key septal components, including PBP1, PBP2, and PBP3.
DISCUSSION

In this study, we show that a broad range of β-lactam antibiotics from three different chemical classes act synergistically with the FtsZ-targeting agent TXA707 against MRSA. Our time-kill synergy results demonstrate that the synergistic actions of the β-lactams in combination with TXA707 are bactericidal in nature. However, the degree of synergistic kill afforded by the β-lactams depends on the specific agent. The question thus arises as to the nature of the correlation, if any, between the magnitude of synergistic kill and the PBP binding affinities and selectivities of the β-lactams. Correlation plots of the PBP binding affinities of the β-lactams (as reflected by the IC$_{50}$ values in Table 1.2) versus their corresponding extents of synergistic kill when combined with TXA707 reveals a clear correlation (R = 0.95) between the degree of bactericidal synergy and affinity for PBP2 (Fig. 1.8B). β-Lactams with a high affinity for PBP2 (low IC$_{50}$) synergize the best with TXA707. No such correlation is evident for the other three PBPs, with R values of 0.56, 0.27, and 0.64 for PBP1, PBP3, and PBP4, respectively (Fig. 1.8A, C, and D). In this connection, PBP2-selective β-lactams (e.g., ceftriaxone, cefotaxime, and ertapenem) are better synergizers with TXA707 than PBP3-selective agents (e.g., cephalaxin and cefradine) (Table 1.2 and Fig. 1.8). That said, β-lactams need not be purely PBP2-selective (e.g., cefidinir, oxacillin, and imipenem) in order to synergize well with TXA707, as long as their PBP binding behavior includes high-affinity targeting of PBP2.

We have previously shown that TXA707 inhibits bacterial cell division by altering the dynamics of FtsZ polymerization and disrupting FtsZ Z-ring formation at midcell (41). The formation of the Z-ring is a critical first step toward formation of the septum in dividing cells (68). Consistent with this critical linkage between Z-ring and septum formation, our TEM results indicate that the FtsZ-targeting actions of TXA707 interfere with the formation of septa in MRSA
cells (Fig. 1.6). The Z-ring serves as a scaffold for the recruitment of essential cell division components to the newly forming septum (18). Among these essential cell division components are the PBPs. Our fluorescence microscopy results reveal that the disruptive actions of TXA707 on septum formation in MRSA cause a mislocalization of key PBPs (likely to be PBP1, PBP2, and PBP3) away from the septum to nonproductive peripheral sites (Fig. 1.7). This observation suggests a potential mechanism underlying the synergy between β-lactams and TXA707. Inhibition of FtsZ function by TXA707 interferes with the septal localization of the PBPs. In so doing, the number of PBPs that are appropriately localized becomes greatly reduced, thereby sensitizing the bacteria to the β-lactams. It is also possible that the actions of the β-lactams on the peripherally localized PBPs adversely impact the integrity of the bacterial cell wall at those peripheral sites, resulting in β-lactam-induced cell lysis and death.

Why then do PBP2-targeting β-lactams synergize the best with TXA707 against MRSA? Pinho and coworkers have demonstrated the cooperative localization and functioning of PBP2 and PBP2a (52, 55). It is likely that mislocalization of PBP2 by the actions of TXA707 results in the concomitant mislocalization of PBP2a. This effect would significantly diminish the molecular basis for the MRSA phenotype, and render MRSA cells particularly susceptible to the dual actions of FtsZ inhibitors and PBP2-targeting β-lactams. Consistent with this notion, previous studies have indicated that FtsZ inhibitors sensitize MRSA to β-lactams to a much greater extent than they do methicillin-susceptible S. aureus (38).

The results of our studies suggest that FtsZ-targeting agents offer an opportunity to repurpose β-lactam antibiotics for use against β-lactam-resistant MRSA strains through combination therapy. Combination therapy with synergistic drug partners has numerous advantages, including a reduced dosage of each drug required for efficacy, a reduced potential for
toxicity, and a reduced potential for the emergence of resistance (72-74). Viewed as a whole, the results of our studies indicate that PBP2-targeting β-lactam antibiotics are optimal synergistic partners with FtsZ-targeting agents for combination therapy of MRSA infections.
Figure 1.1  Fluorescence anisotropy of 1 μM Bocillin as a function of increasing concentrations of PBP1 (A), PBP2 (B), or PBP3 (C). All experiments were conducted at 25 °C in solution containing 100 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 5% (vol/vol) glycerol.
Figure 1.2  (A) Time dependence of the fluorescence anisotropy of 1 μM Bocillin in the presence of the indicated concentrations of PBP4.  (B) Initial fluorescence anisotropy of 1 μM Bocillin as a function of increasing concentrations of PBP4. All experiments were conducted at 25 °C in solution containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 5% (vol/vol) glycerol.
Figure 1.3 (A to C) Fluorescence anisotropy of 1 μM Bocillin in the presence of the indicated β-lactam antibiotic and 2 μM of either PBP1 (A), PBP2 (B), or PBP3 (C). (D) Initial anisotropy velocity of 1 μM Bocillin in the presence of the indicated β-lactam and 50 μM PBP4. The solid lines reflect the nonlinear least-squares fits of the data with equation 1.1, with the exception of the curve for cefepime and PBP3, which could not be fit due to the weak binding of the drug. Experimental conditions were as described in the legends to Figure 1.1 (for PBP1, PBP2, and PBP3) and Figure 1.2 (for PBP4).
Figure 1.4 (A to F) Time-kill curves for MRSA COL showing synergy between TXA707 and cephalexin (A), cefepime (B), oxacillin (C), ceftriaxone (D), imipenem (E), or cefidinir (F). Bacteria were treated with DMSO vehicle (black), β-lactam alone at 0.008× MIC (violet), TXA707 alone at 0.5× MIC (red), or a combination of β-lactam at 0.008× MIC and TXA707 at 0.5× MIC (blue).
Figure 1.5 Change in the log(CFU/mL) at 24 hours \( \{ \log (CFU/mL)_{24} - \log (CFU/mL)_0 \} \) of MRSA COL treated with either a single agent (DMSO vehicle, the indicated \( \beta \)-lactam at 0.008\( \times \)MIC, or TXA707 at 0.5\( \times \)MIC) or with a combination of \( \beta \)-lactam at 0.008\( \times \)MIC and TXA707 at 0.5\( \times \)MIC.
Figure 1.6 Impact of TXA707 alone or in combination with either cefdinir or ceftriaxone on septal formation in MRSA COL. (A to D) TEM micrographs of MRSA COL cells treated for 3 hours with either vehicle (A), TXA707 alone at 8× MIC (B), TXA707 at 1× MIC in combination with cefdinir at 0.008× MIC (C), or TXA707 at 1× MIC in combination with ceftriaxone at 0.008× MIC (D).
**Vehicle**

![Vehicle micrographs]

**TXA707 (8x MIC)**

![TXA707 micrographs]

**Figure 1.7** Impact of **TXA707** on PBP localization in MRSA COL. (A to D) Differential interference contrast (A and C) and fluorescence (B and D) micrographs of MRSA COL cells treated for 3 hours with either vehicle (A and B) or **TXA707** at 8× MIC (C and D). In these micrographs, the PBPs in the cells were labeled by treatment with 2 μM **Bocillinn** for 10 minutes just prior to visualization.
Figure 1.8  (A to D) Correlation plots of the extents to which the β-lactams synergize with TXA707 against MRSA COL versus their affinities (as reflected by the IC_{50} values from Table 1.2) for PBP1 (A), PBP2 (B), PBP3 (C), and PBP4 (D). In these plots, each datum point corresponds to a different β-lactam antibiotic, with the solid lines reflecting linear regression fits of the data. The correlation constant (R value) derived from the linear regression analysis of each data set is indicated.
Table 1.1 Intrinsic activities against MRSA COL.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXA707</td>
<td>2</td>
</tr>
<tr>
<td>Cefradine</td>
<td>128</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>128</td>
</tr>
<tr>
<td>Cefdinir</td>
<td>256</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1024</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2048</td>
</tr>
<tr>
<td>Cefepime</td>
<td>256</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>512</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>512</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>256</td>
</tr>
<tr>
<td>Imipenem</td>
<td>32</td>
</tr>
</tbody>
</table>
Table 1.2 IC$_{50}$ values for the binding of the β-lactam antibiotics to the four native PBPs of *S. aureus*

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>PBP1</th>
<th>PBP2</th>
<th>PBP3</th>
<th>PBP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefradine</td>
<td>516 ± 69</td>
<td>180 ± 7</td>
<td>3.0 ± 0.2</td>
<td>8250 ± 280</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>374 ± 28</td>
<td>154 ± 4</td>
<td>4.7 ± 0.4</td>
<td>8108 ± 304</td>
</tr>
<tr>
<td>Cefepime</td>
<td>1059 ± 38</td>
<td>30.3 ± 1.5</td>
<td>&gt; 7500</td>
<td>2346 ± 39</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>147 ± 15</td>
<td>22.4 ± 1.5</td>
<td>9.2 ± 0.3</td>
<td>392 ± 26</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>42.2 ± 1.7</td>
<td>12.6 ± 0.9</td>
<td>10.4 ± 0.6</td>
<td>1128 ± 90</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>89.7 ± 1.8</td>
<td>7.8 ± 0.5</td>
<td>82.8 ± 6.6</td>
<td>233 ± 7</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>88 ± 3.9</td>
<td>4.2 ± 0.1</td>
<td>531 ± 50</td>
<td>2567 ± 102</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>100 ± 6</td>
<td>3.8 ± 0.1</td>
<td>71.9 ± 5.3</td>
<td>8906 ± 321</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2.7 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>3.9 ± 0.3</td>
<td>85.4 ± 2.4</td>
</tr>
<tr>
<td>Cefdinir</td>
<td>104 ± 4</td>
<td>1.0 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td>126 ± 6</td>
</tr>
</tbody>
</table>

a IC$_{50}$ reflects the concentration of β-lactam antibiotic that reduces the anisotropy (in the case of PBP1, PBP2, and PBP3) or initial anisotropy velocity (in the case of PBP4) of 1 μM Bocillin by 50%. The indicated uncertainties reflect the standard deviation of the experimental anisotropy data points in Figures 1.3 and 1.5S3 from the corresponding fitted curves.
CHAPTER II

IMPACT OF FTSZ INHIBITION ON THE LOCALIZATION OF THE PENICILLIN BINDING PROTEINS IN METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA) is a multidrug-resistant pathogen of acute clinical importance. Combination treatment with an FtsZ inhibitor potentiates the activity of penicillin binding protein (PBP)-targeting β-lactam antibiotics against MRSA. To explore the mechanism underlying this synergistic behavior, we examined the impact of treatment with the FtsZ inhibitor TXA707 on the spatial localization of the five PBP proteins expressed in MRSA. In the absence of drug treatment, PBP1, PBP2, PBP3, and PBP4 localize to the FtsZ Z-ring at mid-cell, contributing to septum formation. By contrast, PBP2a localizes to distinct foci along the cell periphery. Upon treatment with TXA707, cell division becomes disrupted and FtsZ localizes away from mid-cell. PBP1 and PBP3 remain localized with FtsZ, while PBP2 and PBP4 localize away from FtsZ to specific sites in the cell periphery. PBP2a becomes more evenly distributed throughout the cell periphery. We also examined the impact of treatment with the β-lactam antibiotic oxacillin alone and in combination with TXA707 on PBP2a localization. PBP2a localizes to the Z-ring in response to treatment with oxacillin alone, a behavior contributing to the β-lactam resistance of MRSA. Combination treatment with TXA707 causes PBP2a to colocalize with FtsZ in malformed septal-like structures. Our collective results suggest that PBP2, PBP4, and PBP2a may function in cell wall repair and maintenance in response to FtsZ inhibition by TXA707. Upon cotreatment with oxacillin, the localization pattern of PBP2a suggests that it is
no longer able to assist in cell wall repair, thereby rendering the MRSA cells more susceptible to β-lactams.

**INTRODUCTION**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant bacterial pathogen that infects more than 120,000 individuals and causes nearly 20,000 deaths each year in the United States (75). Growing resistance to current standard-of-care antibiotics (5, 8, 10) has highlighted a critical need to develop new antibiotics that can address the MRSA threat (7, 49, 76). Toward this goal, recent efforts have been focused on the development of novel small molecule chemotypes that target bacterial cell division (11, 15, 16, 77, 78). These efforts have resulted in the identification and development of lead compounds that target the essential cell division protein, FtsZ (29-32, 35, 38, 41, 77, 79-82). Treatment of MRSA with such FtsZ inhibitors disrupts bacterial cell division and induces cell death (28, 30-35, 38, 41, 80).

In almost all bacterial species, FtsZ plays a critical role in cell division by acting as a scaffold for recruitment of other components of the cell division machinery (the divisome) (17, 19, 83). FtsZ forms this scaffold by polymerizing at mid-cell and adopting a dynamic ring structure termed the Z-ring (20, 23, 84). Upon formation of the Z-ring, the divisome machinery assembles, with the penicillin binding proteins (PBP s) being among the key proteins that are recruited. These proteins are involved in the synthesis of new cell wall at the septum (46). Methicillin-sensitive *S. aureus* (MSSA) expresses a total of four PBP proteins (PBP1, PBP2, PBP3, and PBP4), of which PBP1 and PBP2 have been identified as essential proteins (60). The PBP proteins are targeted by β-lactam antibiotics, which disable the transpeptidase domains of the proteins and thereby interfere with PBP-induced cross-linking of the bacterial cell wall (43, 44). MRSA expresses an additional PBP (PBP2a) that underlies the resistance of MRSA to most β-lactam antibiotics (53). The affinity
of PBP2a for these β-lactam antibiotics is significantly reduced compared to the other PBPs (85, 86). Co-treatment with FtsZ inhibitors can repurpose β-lactam antibiotics for use against MRSA (34, 37, 38, 42, 79), with such combination treatments being associated with the greatest degree of synergy when the β-lactam targets PBP2 with high affinity (42).

The mechanism underlying the potentiation of β-lactam activity against MRSA by FtsZ inhibitors is unclear. Here we examine the impact of treatment with the FtsZ inhibitor TXA707 (41) on the localization of the five PBPs expressed in MRSA. Toward this goal, we generated a series of MRSA strains expressing fluorescent fusion forms of FtsZ and either PBP1, PBP2, PBP3, or PBP4. We also developed an immunofluorescence approach to monitor the localization of PBP2a. Our results shed light on how FtsZ inhibitors potentiate β-lactam antibiotic activity versus MRSA as well as on the mechanism by which PBP2a facilitates MRSA survival in response to β-lactam antibiotic exposure.
MATERIALS AND METHODS

Bacterial strains and other reagents. *S. aureus* RN4220 (MSSA) and MRSA LAC were provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH. *E. coli* NEB5α was obtained from New England Biolabs. All the MRSA LAC strains expressing fluorescent fusion forms of FtsZ and different PBPs (listed in Table 1) were generated as described below. The PBP2a-specific monoclonal antibody Mouse Anti-MRSA was obtained from RayBiotech (product code: 130-10096-20) and the goat anti-mouse Alexa Flour 488 antibody was from ThermoFisher. Tryptic soy broth (TSB), tryptic soy agar (TSA), cation-adjusted Mueller-Hinton (CAMH) media, and Luria-Bertani (LB) media were obtained from Becton-Dickinson. Ampicillin sodium salt, oxacillin sodium salt, erythromycin, chloramphenicol, isopropyl β-D-1-thiogalactopyranoside (IPTG), Tween 20, bovine serum albumin (BSA), and high-resolution agarose were from Sigma. Phosphate-buffered saline (PBS) was obtained from Lonza. TXA707 was synthesized as previously described (41).

Generation of MRSA LAC strains expressing fluorescent fusion forms of FtsZ and different PBPs. Fluorescent fusion proteins were cloned in MRSA LAC using the general strategy outlined below. The plasmids listed in Table S2 were propagated in *E. coli* NEB5α in the presence of the appropriate antibiotic for each given plasmid. The plasmids were then isolated and purified using the Monarch plasmid miniprep kit (New England Biolabs) and subsequently introduced into electrocompetent *S. aureus* RN4220 cells as previously described (87). Bacteriophage 80α was used to transduce (88) the plasmids from *S. aureus* RN4220 into MRSA LAC-FCh, a MRSA strain that we previously generated (89) containing an ectopic ftsZ-mCherry fusion gene under control of an IPTG-inducible promoter. The MRSA LAC-FCh strains expressing the fluorescent fusion form of sfGFP-PBP1, sfGFP-PBP2, sfGFP-PBP3, or PBP4-sfGFP (LAC-FChP1GFP, LAC-
F<sub>Ch</sub>P2<sub>GFP</sub>, LAC-F<sub>Ch</sub>P3<sub>GFP</sub>, and LAC-F<sub>Ch</sub>P4<sub>GFP</sub>, respectively) were constructed by allelic replacement utilizing the pJB38 vector (90). In each case, DNA fragments P1, P2, and P3 (see Table S3) that contain complementary overhangs were PCR amplified using Q5 High-Fidelity DNA polymerase (New England Biolabs). The resulting amplified fragments were then combined in equimolar quantities with the pJB38 vector, previously linearized by PCR using primers pJB38-F and pJB38-R (Table S4). All four DNA fragments were then joined using the NEBuilder HiFi DNA assembly kit (New England Biolabs), with each resulting construct being verified by sequencing.

The integration of the pJB38 constructs into the MRSA LAC-F<sub>Ch</sub> chromosome and subsequent excision were achieved through a double recombination process, leading to an allelic exchange (90, 91). Briefly, transductants containing the pJB38-derived constructs were maintained at the replication-permissive temperature of 30 °C for plasmid maintenance and confirmation and then frozen down. To initiate recombination, frozen stocks of the transductants were streaked onto TSA plates supplemented with 30 µg/mL chloramphenicol and 10 µg/mL erythromycin and then incubated overnight at 44 °C. Large colonies reflecting clones that had undergone a single recombination event were restreaked onto TSA plates supplemented with 30 µg/mL chloramphenicol and 10 µg/mL erythromycin and then incubated overnight at 44 °C. These single recombinants were then inoculated into 5 mL of TSB and incubated at 30 °C in the absence of chloramphenicol to promote a second round of recombination and subsequent plasmid loss. After consecutive passages over 5 days at 30 °C, the cultures were then serially diluted and plated onto TSA plates supplemented with 100 ng/mL anhydrotetracycline and 10 µg/mL erythromycin. To identify cells that had undergone a second recombination event and subsequent loss of the plasmid, the resulting colonies were replica-patched onto TSA plates supplemented with 10 µg/mL
erythromycin alone, as well as onto TSA plates supplemented with both 10 μg/mL erythromycin and 30 μg/mL chloramphenicol. Chloramphenicol-sensitive colonies were screened by PCR to verify the presence of the appropriate allele utilizing the forward primer for the P1 DNA fragment and the reverse primer for the P3 DNA fragment (listed in Table S3 for each denoted strain). The MRSA LAC strains expressing the fluorescent fusion form of PBP2a-mCherry or mCherry-PBP2a (LAC-P2ACh-1 and LAC-P2ACh-2, respectively) were generated using a similar strategy to that described above, with the exception that wild-type MRSA LAC was used as the recipient strain instead of MRSA LAC-FCh.

**Time-dependent growth assay.** An exponentially growing culture of each MRSA strain was diluted in CAMH broth to a final count of 5 x 10^5 CFU/mL. The CFU/mL of each culture at time zero was verified by plating serial dilutions in duplicate on TSA plates. The cultures were then incubated at 37°C with shaking. The CFU/mL in each culture was determined over time by withdrawing samples at time points ranging from 3 to 24 hours and then plating appropriate serial dilutions onto TSA plates. All TSA plates were incubated at 37°C for 24 hours and the CFU/mL at each time point determined.

**Minimal inhibitory concentration (MIC) assay.** MIC assays were conducted in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution (92). Briefly, log-phase MRSA cells were added to 96-well microtiter plates (at 5 x 10^5 CFU/mL) containing 2-fold serial dilutions of TXA707, oxacillin, or vancomycin in CAMH broth, with each concentration of antibacterial agent being present in duplicate. The final volume in each well was 0.1 mL, and the microtiter plates were incubated aerobically for 18 hours at 37°C. Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) using a SpectraMax
M2 plate reader (Molecular Devices, Inc.), with the MIC being defined as the lowest compound concentration at which growth was ≥90% inhibited.

**Differential interference contrast (DIC) and fluorescence microscopy.** Each MRSA strain was grown to log-phase in 5 mL of TSB supplemented with 10 µg/mL erythromycin and then diluted to an OD<sub>600</sub> of 0.1 in 5 mL of TSB supplemented with 10 µg/mL erythromycin. The log-phase cultures were then treated with DMSO vehicle, 4 µg/mL **TXA707** (4x MIC), and 10 nM IPTG for 3 hours at 37 °C. Each culture was then centrifuged at 15,000 x g for 1 minute and washed twice with 1 mL of PBS. Cells were then resuspended in 200 µL of PBS. 8 µL of this final cell suspension were spread on a 0.25 mm layer of 1.5% high-resolution agarose in PBS, which was mounted on a petrographic 27 x 46 x 1.2 mm microscope slide (Ward’s Natural Science) using a 1.7 x 2.8 x 0.025 cm Gene Frame (ThermoFisher). A 24 x 40 mm cover slip (Azer Scientific) was then applied to the agarose pad in preparation for microscopic visualization.

All DIC and fluorescence microscopy experiments were conducted using a Total Internal Reflection Fluorescence (TIRF) microscope that was custom-built on the basis of a commercial inverted microscope (Ti-E, Nikon) (93). The microscope was equipped with a high NA objective lens (CFI-apo 100X, NA 1.49, Nikon), an sCMOS camera (Zyla 4.2, Andor), and the 488 nm Genesis MX488-1000 STM and 561 nm Genesis MX561-1000 STM excitation lasers (Coherent). For DIC imaging, a white light LED (LDB101F, Prior) was used along with Nikon’s DIC modules. sfGFP was imaged using 488 nm laser excitation and a green emission band pass filter (ET525/50m, Chroma), while mCherry was imaged using 561 nm laser excitation and an orange emission band pass filter (ET605/52m, Chroma). Multi-channel images were obtained by triggered-acquisition schemes, using AOTF (AOTFnC-400.650-TN, Quanta-Tech), TTL signal out of the sCMOS camera, a Data Acquisition Card (PCIe-7852R, NI), and the Nikon NIS
Elements software. Extra magnification was achieved using a built-in 1.5X intermediate magnification changer on the microscope. MRSA samples were first inspected in the DIC channel and then switched to the fluorescence channel to adjust the focus as well as the excitation laser tilt angle to the optimal TIRF imaging condition. For imaging sfGFP, the 488 nm laser was used at power settings in the range of 2 to 15 mW, coupled with exposure times ranging from 80 to 400 msec. For imaging mCherry, the 561 nm laser was used at power settings in the range of 20 to 40 mW, coupled with exposure times ranging from 200 to 700 msec. The Perfect Focus System (Nikon) was used to actively stabilize focus drift while acquiring images.

**Characterization of the specificity of the mouse anti-MRSA monoclonal antibody.** *E. coli* BL21 (DE3) strains expressing recombinant forms of PBP1, PBP2, PBP3, or PBP4 from *S. aureus* (SaPBP1, SaPBP2, SaPBP3, and SaPBP4, respectively) were generated as described previously (42). *S. aureus* PBP2a (SaPBP2a) was cloned and expressed in *E. coli* BL21 (DE3) as detailed in the Supplementary Material. For the expression of recombinant SaPBP1, SaPBP2, SaPBP3, SaPBP4, and SaPBP2a, each *E coli* strain was cultured overnight at 37 °C in LB broth and then diluted 1:100 into 10 mL of LB broth. These cultures were then grown at 37 °C to an OD 600 of 0.3, whereupon PBP expression was induced by the addition of 1 mM IPTG and subsequent incubation for 3 hours at 37 °C. Overnight cultures of MRSA LAC and MSSA RN4220 cells were diluted 1:10 into in TSB and grown for 3 hours at 37 °C. The MRSA and MSSA cells were then lysed by addition lysostaphin (Sigma) at a concentration of 1 mg/mL and subsequent incubation for 1 hour at 37 °C.

All the *E. coli*, MRSA, and MSSA samples were then washed twice with 500 µL of PBS and resuspended in 2 mL of PBS. The cells were sonicated for 5 minutes at 0 °C using a Qsonica Q500 sonicator (equipped with a 1/8-inch probe) with an on/off cycle of 10 seconds and an
amplitude set at 10%. The total protein concentration in each cell lysate was then quantified using a Pierce BCA Protein Assay Kit (ThermoFisher). A 12% SDS-PAGE gel (Biorad) was loaded with 0.5 µg/mL of purified recombinant SaPB2a and 500 µg/mL total protein from each cell lysate. Western blotting was conducted using standard procedures, followed by incubation with the mouse anti-MRSA antibody (RayBiotech) at a 1:1,000 dilution. Secondary antibody treatment was performed with the goat anti-mouse IgG HRP (ThermoFisher) at a 1:100,000 dilution. The western blot was developed using SuperSignal West Pico PLUS (ThermoFisher) solutions and visualized by chemiluminescence using an Amersham Imager 680.

**Immunofluorescence microscopy.** MRSA LAC-Fch cells were grown to log-phase in 5 mL of TSB supplemented with 10 µg/mL erythromycin and then diluted to an OD<sub>600</sub> of 0.1 in 5 mL of TSB supplemented with 10 µg/mL erythromycin. The log-phase cultures were then supplemented with 10 nM IPTG and treated for 3 hours at 37 °C with either DMSO vehicle, 0.5 µg/mL TXA707 (1/2x MIC), 4 µg/mL TXA707 (4x MIC), 2 µg/mL oxacillin (1/32x MIC), or a combination of 0.5 µg/mL TXA707 and 2 µg/mL oxacillin. Each culture was then centrifuged at 15,000 x g for 1 minute and washed twice with 1 mL of PBS. Cells were then resuspended in 500 µL of PBS containing 2.4% (v/v) formaldehyde, followed by incubation for 15 minutes at room temperature and then 25 minutes on ice. Each culture was then centrifuged at 15,000 x g for 1 minute and washed twice with 1 mL of PBS containing 0.01% Tween 20. Cells were then resuspended in 500 µL of GTE buffer containing 50 mM glucose, 20 mM Tris-HCl (pH 7.6), and 10 mM EDTA. 100 µL of the resulting suspension was then mounted on a poly-L-Lysine-coated, 18 x 18 mm cover slip (VWR). 200 µL of 30 ng/µL lysostaphin was then applied and allowed to equilibrate for 1 minute. Cells were then washed three times with GTE buffer, air dried, and subsequently rehydrated by applying 200 µL of PBS. After equilibration for 5 minutes, the cells were then
blocked for 45 minutes using 300 µL of 2% (w/v) BSA. PBP2a was detected via immunofluorescence using the PBP2a-specific mouse anti-MRSA monoclonal antibody (RayBiotech). Serial two-fold dilutions of antibody (ranging from 1/100 to 1/1600) were added to each sample and incubated overnight at 4 °C. The cells were then washed 8 times with PBS and incubated in the dark for 2 hours with a goat anti-mouse Alexa Flour 488 antibody (ThermoFisher) diluted 3/1000 with PBS containing 2% (w/v) BSA. Cells were again washed 8 times with PBS. Aqua-Poly mounting medium (Polysciences) was then added and the cells were mounted on a petrographic 27 x 46 x 1.2 mm microscope slide (Ward’s Natural Science). The slides were then visualized by DIC and fluorescence microscopy as described above.

**Transmission electron microscopy (TEM).** Log-phase MRSA LAC-FCh cells were diluted to an OD$_{600}$ of 0.1 in 5 mL of TSB and then cultured at 37 °C for 3 hours in the presence of DMSO vehicle, 0.5 µg/mL **TXA707** (1/2x MIC), 2 µg/mL **oxacillin** (1/32x MIC), or a combination of 0.5 µg/mL **TXA707** and 2 µg/mL **oxacillin**. The cultures were then centrifuged at 16,000 x g for 3 minutes at room temperature. The resulting bacterial pellets were washed with 1 mL of PBS and then resuspended in 500 µL of 100 mM cacodylate buffer (pH 7.2) containing 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde. The samples were then prepared for TEM acquisition and the micrographs acquired as previously described (42).
RESULTS

Impact of the genetic modifications on the growth and antibiotic sensitivity profile of MRSA LAC. After constructing our genetically modified MRSA LAC strains, we first sought to explore whether our genetic alterations affected cell fitness and antibiotic sensitivity. To this end, we monitored the time-dependent growth of each genetically modified strain and compared the resulting growth curves to that of the wild-type LAC strain. Significantly, the growth rates of the genetically modified strains were similar to that of the wild-type strain (Fig. 2.S1), confirming that our genetic alterations did not alter the fitness of the MRSA cells. We also examined the sensitivity of our genetically modified strains to treatment with TXA707, vancomycin, and oxacillin, as well as with the cephalosporins ceftriaxone, cefotaxime, and cephalaxin. All six agents were associated with similar minimal inhibitory concentrations (MICs) against wild-type MRSA LAC and strains expressing FtsZ-mCherry (LAC-FCh) alone or both FtsZ-mCherry and either sfGFP-PBP1 (LAC-FChP1GFP), sfGFP-PBP2 (LAC-FChP2GFP), sfGFP-PBP3 (LAC-FChP3GFP) or PBP4-sfGFP (LAC-FChP4GFP) (see Table S1), indicating that these genetic alterations had no impact on antibiotic sensitivity. By contrast, MRSA LAC cells expressing either PBP2a-mCherry (LAC-P2ACh-1) or mCherry-PBP2a (LAC-P2ACh-2) lost their resistance to oxacillin, ceftriaxone, cefotaxime, and cephalaxin, with the MIC for oxacillin, ceftriaxone, cefotaxime, and cephalaxin against these strains being 0.125, 4, 2, and 4 μg/mL, respectively (compared to corresponding MIC values of 64, 512, 128, and 128 μg/mL against the wild-type strain). Significantly, the MIC values determined for oxacillin, ceftriaxone, cefotaxime, and cephalaxin against the MRSA LAC-P2ACh-1 and LAC-P2ACh-2 strains are comparable to those determined for the four b-lactam antibiotics against the MSSA RN4220 strain (Table S1). Thus, the PBP2a-mCherry and mCherry-PBP2a proteins were non-functional, transforming the MRSA strains expressing these conjugate proteins into MSSA strains. These observations are consistent with previous studies by the Pinho
group indicating that appending amino acids to the C- or N-terminus of PBP2a was deleterious to the localization and function of the protein (94). We therefore used immunofluorescence for visualizing the localization of PBP2a (as detailed below).

**Impact of TXA707 treatment on MRSA cell morphology and the localization of FtsZ.** We sought to determine the impact of TXA707 treatment on FtsZ localization in MRSA LAC-Fch cells expressing FtsZ-mCherry. For this purpose, cells were treated with either vehicle (DMSO) or 4 μg/mL TXA707 (4x MIC) for 3 hours prior to visualization by Total Internal Reflection Fluorescence (TIRF) microscopy. Vehicle-treated cells (n = 641) maintained a normal average diameter of approximately 0.84 ± 0.07 μm (Figs. 2.1A,H). Among the 641 vehicle-treated cells analyzed, FtsZ is localized to the septum at mid-cell in 44% of them (Fig. 2.1I). In 16% of the analyzed cells, FtsZ forms visible Z-rings (as shown in Figs. 2.1B,C and schematically depicted in Fig. 2.1G). The remainder of the analyzed cells have FtsZ localized to the cell periphery (25%) or in a diffuse pattern throughout the cell (14%).

In marked contrast to vehicle-treated cells, cells treated with TXA707 (n = 349) almost double in size, with an average diameter of 1.50 ± 0.24 μm (Figs. 2.1D,H). Moreover, FtsZ is no longer predominantly localized to the septum at mid-cell, with only 6% of the analyzed cells exhibiting this phenotype compared to 44% of vehicle-treated cells (Fig. 2.1I). Instead, 43% of the TXA707-treated cells have FtsZ localized in a diffuse pattern throughout the cell (compared to only 14% of vehicle-treated cells with this phenotype). In addition, 39% of the 349 TXA707-treated cells analyzed exhibit a phenotype in which FtsZ adopts multiple ring-shaped structures inside the cell (shown in Figs. 2.1E,F and schematically depicted in Fig. 2.1G), with an average diameter of 0.52 ± 0.12 μm. Thus, treatment with TXA707 increases the percentage of cells in which FtsZ has formed visible ring-shaped structures by 2.4-fold (from 16% to 39%) relative to
vehicle treatment (Fig. 2.1I). Relative to vehicle, treatment with **TXA707** does not induce a significant change in the percentage of cells in which FtsZ is localized to the cell periphery (from 25% to 27%).

**Impact of TXA707 treatment on the localization of FtsZ relative to that of PBP1, PBP2, PBP3, PBP4, or PBP2a in MRSA.** To understand the impact of FtsZ inhibition on the localization of the five PBPs in MRSA, we generated MRSA LAC strains expressing both FtsZ-mCherry as well as sfGFP fusion proteins of PBP1, PBP2, PBP3, and PBP4 (LAC-FChP1GFP, LAC-FChP2GFP, LAC-FChP3GFP, LAC-FChP4GFP, respectively). In addition, we also developed an immunofluorescence approach to monitor the impact of FtsZ inhibition on the localization of PBP2a in the LAC-FCh strain. The sections that follow describe our resulting characterizations.

**PBP1:** Our initial characterizations explored the impact of TXA707 treatment on MRSA LAC cells expressing FtsZ-mCherry and sfGFP-PBP1 (LAC-FChP1GFP) at its native locus. In vehicle-treated LAC-FChP1GFP cells \( n = 919 \), PBP1 and FtsZ are localized to the septum at mid-cell in 42% and 59% of the cells, respectively (see Figs. 2.2B-D,I,J). Most of the non-septally localized PBP1 and FtsZ are localized to the cell periphery, with these phenotypes occurring in 49% and 29% of the analyzed cells, respectively (Fig. 2.2J). The remainder of the vehicle-treated cells have PBP1 and FtsZ localized in a more diffuse pattern (9% of the cells in the case of PBP1 and 13% of cells in the case of FtsZ). Significantly, PBP1 and FtsZ colocalize in the majority (70%) of the vehicle-treated LAC-FChP1GFP cells (Fig. 2.2J), with much of this colocalization occurring at the septum and FtsZ Z-ring structures (as depicted in Figs. 2.2D,I).

Upon treatment of LAC-FChP1GFP cells \( n = 535 \) with **TXA707**, the average cell diameter increases approximately 2.1-fold from 0.84 ± 0.09 to 1.77 ± 0.27 µm (Figs. 2.2A,E and Supplemental Fig. 2.S2), consistent with our observations described above for LAC-FCh cells.
Moreover, TXA707 treatment markedly reduces the percentage of LAC-FChP1GFP cells exhibiting septally-localized PBP1 from 49% to 2% (Fig. 2.2J). Instead, PBP1 localizes to distinct foci across the cell periphery in the majority (59%) of the TXA707-treated cells analyzed (Figs. 2.2F,J). Interestingly, FtsZ appears to co-localize to those same peripheral foci (Figs. 2.2G-I). Relative to vehicle, TXA707 treatment is also associated with a significant increase in the percentage of cells exhibiting a diffuse localization phenotype for both PBP1 (from 9% to 39%) and FtsZ (from 13% to 45%) (Fig. 2.2J). In all, PBP1 and FtsZ colocalize in 65% of the TXA707-treated cells, a behavior consistent with the two proteins undergoing a similar pattern of mislocalization in response to the FtsZ inhibitor.

**PBP3:** We next explored the impact of TXA707 treatment on MRSA LAC cells expressing FtsZ-mCherry and sfGFP-PBP3 (LAC-FChP3GFP) at its native locus. A total of 545 vehicle-treated and 326 TXA707-treated LAC-FChP3GFP cells were analyzed. This analysis revealed similar effects on cell diameter (Supplemental Fig. 2.S2) as well as on PBP3 and FtsZ localization phenotype (Fig. 2.3) to those observed in response to TXA707 treatment of LAC-FChP1GFP cells. This behavior included marked TXA707-induced reductions in the septal localization of both PBP3 (from 44% to 3% of cells) and FtsZ (from 58% to 3% of cells), coupled with significant colocalization (in 60% of TXA707-treated cells) of both PBP3 and FtsZ present in diffuse and peripheral localization phenotypes (Fig. 2.3H-J). These collective results suggest that the FtsZ inhibitor induces a similar pattern of mislocalization between FtsZ and not only PBP1, but also PBP3.

**PBP2:** We also examined the impact of TXA707 treatment on MRSA LAC cells expressing FtsZ-mCherry and sfGFP-PBP2 (LAC-FChP2GFP) at its native locus, with a total of 1,071 vehicle-treated cells and 368 TXA707-treated cells being analyzed. The pattern of observed
changes in cell diameter (Figs. 2.4A,E and Supplemental Fig. 2.S2) and FtsZ localization (Figs. 2.4C,G,I,J) in response to **TXA707** treatment were similar to those described above in our studies of LAC-Fchp1GFP and LAC-Fchp3GFP cells. In vehicle-treated LAC-Fchp2GFP cells, PBP2 localizes to the septum at mid-cell in 38% of the analyzed cells and to the cell periphery in 57% of the cells (Figs. 2.4B,F,J). Very few (2%) of the vehicle-treated cells analyzed exhibit a phenotype in which PBP2 localizes in a diffuse pattern (Fig. 2.4J). PBP2 colocalizes with FtsZ in just over 2/3 (69%) of the vehicle-treated cells (Figs. 2.4D,I,J). Treatment with **TXA707** induces a significant decrease (from 38% to 0.2%) in cells exhibiting septally localized PBP2 and a concomitant increase (from 2% to 45%) in cells exhibiting a diffuse PBP2 phenotype in which multiple foci of PBP2 are distributed throughout large regions of the cell (Figs. 2.4F,I,J). The percentage of cells in which PBP2 is localized to foci along the cell periphery does not change significantly upon **TXA707** treatment (Fig. 2.4J). While PBP2 and FtsZ colocalize in a significant percentage (69%) of vehicle-treated cells, the two proteins colocalize in only 35% of **TXA707**-treated cells (Figs. 2.4D,H-J). Thus, contrary to our observations described above for PBP1 and PBP3, the mislocalization of PBP2 in response to **TXA707** treatment appears independent of FtsZ mislocalization. A similar behavior for PBP2 was previously reported by Tan *et al.* upon treatment with the FtsZ inhibitor PC190723 (34).

**PBP4:** We next characterized the effects of vehicle relative to **TXA707** treatment on MRSA LAC cells (*n* = 300 and 326, respectively) expressing FtsZ-mCherry and sfGFP-PBP4 (LAC-Fchp4GFP) at its native locus. The overall impact of **TXA707** treatment on LAC-Fchp4GFP cell diameter (Figs. 2.5A,E and Supplemental 2.S2) and FtsZ localization (Figs. 2.5C,G,I,J) was similar to that observed in LAC-Fchp1GFP, LAC-Fchp2GFP, and LAC-Fchp3GFP cells. Like PBP1, PBP2, and PBP3, PBP4 localizes to the septum in a significant percentage (55%) of vehicle-treated
cells, while localizing in a more diffuse pattern in a much smaller percentage (11%) of cells (Figs. 2.5B,I,J). PBP4 localizes to the cell periphery in approximately 1/3 (33%) of the vehicle-treated cells (Fig. 2.5J). The extent to which PBP4 colocalizes with FtsZ in vehicle-treated cells is significant, with 75% of the analyzed cells exhibiting this phenotype (Figs. 2.5D,I,J).

Upon TXA707 treatment, PBP4 relocalizes away from the septum to distinct foci along the cell periphery (Figs. 2.5F,I,J). In this connection, the percentage of cells exhibiting the septal PBP4 phenotype decreases from 55% to 0.9%, while the percentage of cells exhibiting the peripheral PBP4 phenotype increases from 33% to 89% (Fig. 2.5J). TXA707 treatment is also associated with a marked reduction in the observed colocalization of PBP4 and FtsZ, with the percentage of cells exhibiting this phenotype decreasing from 75% in vehicle-treated cells to 15% in TXA707-treated cells. Viewed as a whole, our characterizations of LAC-FChP1GFP, LAC-FChP2GFP, and LAC-FChP3GFP, and LAC-FChP4GFP suggest that the mislocalization of FtsZ in response to treatment with the FtsZ inhibitor coincides with the mislocalization of PBP1 and PBP3 but appears independent of the mislocalization of PBP2 and PBP4.

PBP2a: We next explored the impact of TXA707 treatment on the relative localization of PBP2a and FtsZ in MRSA LAC cells expressing FtsZ-mCherry (LAC-FCh). We used an anti-MRSA monoclonal antibody and immunofluorescence to visualize PBP2a in these studies, since fluorescent fusion forms of PBP2a were non-functional. Prior to utilizing the antibody in our immunofluorescence microscopy experiments, we verified its selectivity for PBP2a by Western blot analysis using purified PBP2a as well as cell lysates of MRSA LAC, MSSA RN4220, and E. coli BL21 (DE3) engineered to express S. aureus PBP1, PBP2, PBP3, PBP4, or PBP2a. Significantly, this analysis (the results of which are shown in Supplemental Fig. 2.S3) confirmed
the selectivity of the anti-MRSA antibody for PBP2a, with no observed cross-reactivity with PBP1, PBP2, PBP3, or PBP4.

Fig. 2.6 shows the results of our immunofluorescence microscopy studies probing the impact of TXA707 on the localization of PBP2a and FtsZ in LAC-Fch cells (n = 536 for vehicle treatment and 315 for TXA707 treatment). In marked contrast to the behavior of PBP1, PBP2, PBP3, and PBP4 in vehicle-treated cells, PBP2a does not localize to the septum at mid-cell, with none of the analyzed cells exhibiting this phenotype (Figs. 2.6B,I,J). Instead, PBP2a localizes to distinct foci across the cell periphery in the vast majority (85%) of vehicle-treated cells and in a more diffuse pattern in the remaining cells. As expected, FtsZ localizes to the septum in a significant percentage (48%) of vehicle-treated cells, while localizing to foci in the cell periphery or in a diffuse pattern in 22% and 30% of the cells, respectively (Figs. 2.6C,I,J). Significantly, none of the 536 vehicle-treated cells analyzed exhibit a phenotype in which FtsZ and PBP2a are colocalized (Figs. 2.6D,I,J).

In response to TXA707 treatment, PBP2a localizes to an increased number of foci along the periphery of the enlarged cell (Figs. 2.6F,I), with the overall percentage of cells exhibiting peripherally localized PBP2a (81%) being similar to the 85% observed with vehicle treatment (Fig. 2.6J). As expected, the prevalence of cells in which FtsZ is localized to the septum decreases markedly (from 48% to 8%) with TXA707 treatment (Figs. 2.6C,G,I,J). TXA707 treatment is also associated with FtsZ localization to foci in the cell periphery in 62% of the cells (Fig. 2.6J). As observed with vehicle treatment, none of the 315 TXA707-treated cells analyzed exhibit a phenotype in which FtsZ and PBP2a are colocalized (Figs. 2.6H,I,J). Even when both proteins are localized to the cell periphery, FtsZ and PBP2a appear to form foci distinct from one another (as exemplified by the cell denoted with arrow 2 in Figs. 2.6F-I). Like the behavior noted above for
PBP2 and PBP4, the relocalization of PBP2a resulting from TXA707 treatment appears to be independent of the corresponding relocalization of FtsZ.

**Impact of treatment with the synergistic combination of TXA707 and oxacillin on cell morphology as well as on the localization of FtsZ, PBP2a, and PBP2.** FtsZ inhibitors have been shown to sensitize MRSA to β-lactam antibiotics, resulting in a synergistic antibacterial effect (34, 37, 38, 42, 79). We sought to examine how treatment of MRSA LAC-Fch cells with a sub-MIC concentration of TXA707 alone, oxacillin alone, or a combination of both TXA707 and oxacillin impacts cell morphology as well as the localization of FtsZ and PBP2a.

We first compared cells treated with 2 µg/mL (1/32x MIC) oxacillin alone (n = 835) relative to cells treated with vehicle (n = 535). Oxacillin-treated cells are associated with an average diameter of 1.30 ± 0.17 µm, a 1.5-fold increase relative to that (0.86 ± 0.13 µm) associated with vehicle-treated cells (Figs. 2.7A and 2.8A). In response to oxacillin treatment, PBP2a localizes to the septum at mid-cell (as exemplified by the cell denoted with arrow 2 in Figs. 2.7B,M) in 15% of the 835 cells analyzed (Fig. 2.8B). Significantly, this behavior markedly contrasts that associated with vehicle treatment, where none of the 535 analyzed cells exhibit septally localized PBP2a (Fig. 2.8B). Thus, treatment with oxacillin appears to induce the relocalization of PBP2a to the septum in a statistically significant percentage of cells. In addition, we observe oxacillin-treated cells in which PBP2a is localized to specific sites around the cell periphery (as indicated by the cell denoted with arrow 1 in Figs. 2.7B,M), although the prevalence of this PBP2a phenotype is markedly reduced (from 85% to 25%) relative to vehicle-treated cells (Fig. 2.8B). PBP2a localizes in a more diffuse pattern in 60% of oxacillin-treated cells, a rise of 45% compared to the vehicle treatment condition (Fig. 2.8B). FtsZ localization to the septum is reduced in response to oxacillin treatment, with a concomitant increase in the diffuse FtsZ
phenotype (Figs. 2.7C,M and 2.8B). Significantly, in striking contrast to the absence of colocalization in vehicle treated cells, FtsZ colocalizes with PBP2a in 10% of the 835 oxacillin-treated cells analyzed (Fig. 2.8B), with this colocalization occurring almost exclusively in cells where both PBP2a and FtsZ are septally localized (as indicated by the cell denoted with arrow 2 in Figs. 2.7D,M).

Treatment with 0.5 µg/mL (1/2x MIC) TXA707 alone results in enlarged cells (average diameter = 1.52 ± 0.34 µm) (Fig. 2.8A), some with oblong shapes (Figs. 2.7E,M), a behavior similar to that observed upon treatment with 4 µg/mL TXA707 (Figs. 2.6E,M). Interestingly, Pinho and coworkers observed a similar oblong-shaped morphology in untreated MRSA cells expressing a FtsZ variant containing the G193D mutation (95). The localization patterns for FtsZ and PBP2a observed upon treatment with 0.5 µg/mL TXA707 (Figs. 2.7F-H) were comparable to those observed with 4 µg/mL TXA707 treatment, with very few cells exhibiting a septal PBP2a phenotype or colocalization between PBP2a and FtsZ (Fig. 2.8B).

Remarkably, co-treatment with a combination of both 2 µg/mL oxacillin and 0.5 µg/mL TXA707 yields unique morphological changes and localization patterns for both FtsZ and PBP2a relative to those observed upon treatment with either agent alone. Specifically, the combination treatment causes many cells to adopt oblong shapes (Figs. 2.7I-M), though smaller in size (average diameter = 1.17 ± 0.24 µm) than those observed with TXA707 treatment alone (Fig. 2.8A). The smaller size of the combination-treated cells relative to the cells treated with 0.5 µg/mL TXA707 alone may reflect the synergistic bactericidal activity of oxacillin in combination with TXA707, with this combination inducing a greater rate of kill than either agent alone (42). This enhanced bactericidal activity may preclude the combination-treated cells from ever attaining the size induced by treatment with TXA707 alone. In 52% of the 239 combination-treated cells analyzed
(Fig. 2.8B), PBP2a appears to localize into distinct curved structures across the cell periphery (as indicated by the cells denoted with arrows 5 and 6 in Figs. 2.7J,M), while also localizing to the septum in 24% of the combination-treated cells. FtsZ colocalizes with PBP2a in many (38%) of these cells (Figs. 2.7L,M and 2.8B).

To gain further insight into the behavior of MRSA LAC-Fch cells in response to co-treatment with the synergistic combination of oxacillin and TXA707, we sought to correlate our DIC and fluorescence microscopy results shown in Fig. 2.7 with corresponding transmission electron microscopy (TEM) studies, from which representative results are shown in Fig. 2.9. Cells treated with DMSO vehicle divide normally, forming well-defined septa at mid-cell (denoted with the arrow in Fig. 2.9A). Treatment with 0.5 µg/mL (1/2x MIC) TXA707 alone causes the cells to enlarge significantly and disrupts the ability of the cells to generate a normal septum (Fig. 2.9B). Instead, TXA707-treated cells appear to undergo multiple attempts at generating a septal structure but are unable to complete the fully formed septum (highlighted by the white arrows Fig. 2.9B). Aberrant attempts at division in TXA707-treated cells can also result in the formation of a blebs (as highlighted by the yellow arrow in Fig. 2.9B). Cells treated with 2 µg/mL (1/32x MIC) oxacillin alone appear larger in size than vehicle-treated cells but are still able to form septa (as indicated by the arrow in Fig. 2.9C). However, the septa in oxacillin-treated cells appear thicker than those in vehicle-treated cells (compare Figs. 2.9A,C). Co-treatment with the combination of 0.5 µg/mL TXA707 and 2 µg/mL oxacillin yields highly blebbed and oblong-shaped cells with multiple thick and curved irregular septal-like structures (as highlighted by the arrows in Fig. 2.9D), similar in nature to the curved PBP2a structures observed in our immunofluorescence micrographs of MRSA LAC-Fch cells treated with the same combination of agents (Figs. 2.7J,M).
We also examined the impact of treatment with 2 µg/mL oxacillin alone or in combination with 0.5 µg/mL TXA707 on the relative localization of PBP2 and FtsZ in MRSA LAC-FChP2GFP cells. Both treatment conditions result in FtsZ localization patterns (Fig. 2.S4) similar to those observed in our immunofluorescence studies of MRSA LAC-FCh cells (Figs. 2.7 and 2.8). PBP2 localizes to the septum in 33% of the cells (n = 406) treated with oxacillin alone (Fig. 2.S4B,I,K). This behavior is consistent with that previously observed by Pinho and coworkers, who demonstrated that β-lactam treatment yields septal localization of PBP2 in MRSA but not MSSA cells (55). PBP2 also localizes to foci in the cell periphery in 54% of the oxacillin-treated cells analyzed, with the prevalence of both peripheral and septal PBP2 being similar in oxacillin-treated relative vehicle-treated cells (Fig. 2.S4). PBP2 colocalizes with FtsZ in 51% of the oxacillin-treated cells (Figs. 2.S4D,K), compared to 69% in the vehicle treatment condition. Relative to treatment with oxacillin alone, treatment of MRSA LAC-FChP2GFP cells (n = 432) with a combination of both 2 µg/mL oxacillin and 0.5 µg/mL TXA707 results in a significant reduction (from 33% to 11%) in the prevalence of PBP2 localized to the septum coupled with an increase (from 54% to 72%) in the prevalence of PBP2 localized to foci in the cell periphery (Fig. 2.S4). Colocalization of PBP2 and FtsZ is also markedly reduced (from 51% to 28%) with combination relative to oxacillin treatment (Figs. 2.S4D,H,I,K).
DISCUSSION

FtsZ and the PBPs are key components of the divisome machinery that play important roles in septum formation and cell division (20, 46, 96). FtsZ inhibitors have been shown to act synergistically with PBP-targeting β-lactam antibiotics against MRSA, effectively resensitizing MRSA to the β-lactams (34, 37, 38, 42, 79). To further our understanding of the basis for this behavior, we examined the impact of the FtsZ inhibitor TXA707 on the localization of FtsZ and the five PBPs in MRSA (PBP1, PBP2, PBP3, PBP4, and PBP2a). To this end, we genetically engineered MRSA LAC to express a FtsZ-mCherry fusion protein and a sfGFP fusion form of PBP1, PBP2, PBP3 or PBP4. We also developed an immunofluorescence approach for monitoring the localization of PBP2a in MRSA LAC expressing FtsZ-mCherry.

In MRSA cells treated with DMSO vehicle, PBP1, PBP2, PBP3, and PBP4 are recruited to FtsZ Z-rings formed at mid-cell (Figs. 2.2-5), consistent with previous reports suggesting that all four PBPs are involved in cell wall synthesis at the septum during cell division (27, 61, 62, 64, 97, 98). In striking contrast, PBP2a does not localize to FtsZ Z-rings at mid-cell, but rather localizes to distinct foci around the cell periphery (Fig. 2.6). This observation suggests that under vehicle-treated conditions, PBP2a is not involved in new cell wall synthesis at the septum. Instead, it may be functioning in cell wall maintenance and repair around the cell periphery. García-Fernández et al. have demonstrated that PBP2a is localized in lipid rafts in MRSA cell membranes (99). This observation coupled with our results suggests that the PBP2a may be localized in depots around the cell periphery where it is available to act in response to conditions encountered by the cell that compromise the cell wall.

Upon treatment with TXA707, septum formation and cell division are disrupted, with FtsZ and PBP1, PBP2, PBP3 and PBP4 being mislocalized away from mid-cell (Figs. 2.2-5). PBP1
and PBP3 become mislocalized to distinct foci across the cell periphery, with FtsZ co-localizing to these same foci (Figs. 2.2 and 2.3). This co-localization suggests that PBP1 and PBP3 may function in maintaining septal integrity through direct or indirect linkage with FtsZ. In contrast to the behavior of PBP1 and PBP3, PBP2 and PBP4 do not co-localize with FtsZ in response to FtsZ inhibition with TXA707. Instead, PBP2 and PBP4 are localized to foci across the cell periphery that are distinct from those to which FtsZ has localized (Figs. 2.4 and 2.5). Pinho and Errington observed a similar behavior for PBP2 in methicillin-sensitive S. aureus (MSSA) cells in which cell division had been disrupted through depletion of FtsZ (100). The authors speculated that PBP2 plays a major role in the cell wall remodeling and repair necessitated by the enlarged cell resulting from the block of cell division. Our results suggest that both PBP2 and PBP4 are involved in such a cell wall remodeling and repair process induced by FtsZ inhibition with TXA707. Interestingly, this process does not appear to be coordinated by FtsZ.

In response to treatment with TXA707, PBP2a becomes more evenly distributed around the cell periphery, while also forming particularly concentrated foci in distinct locations (Fig. 2.6). This behavior suggests that PBP2a may play a similar role to PBP2 and PBP4 in response to FtsZ inhibition, a function geared toward cell wall remodeling, maintenance, and repair necessitated by a disruption of cell division. Łęski and Tomasz have previously demonstrated that PBP2, PBP4 and PBP2a act cooperatively during cell wall synthesis in MRSA (97). The similar behaviors we observe for PBP2, PBP4 and PBP2a upon FtsZ inhibition with TXA707 are consistent with the three PBPs having cooperative roles in cell wall maintenance and repair in the enlarging cells.

To further explore the mechanism of synergy between FtsZ inhibitors and β-lactam antibiotics against MRSA, we investigated the impact of treatment with sub-MIC concentrations of TXA707 alone, oxacillin alone, or a combination of both agents on the localization of FtsZ and
PBP2a. Upon treatment with oxacillin alone at 1/32x MIC, PBP2a relocalizes to the FtsZ Z-ring at mid-cell (Figs. 2.7 and 2.8), suggesting that PBP2a can indeed participate in new cell wall formation at the septum, but only in the presence of a β-lactam antibiotic. This seminal observation provides an indication of how PBP2a is able to confer MRSA with resistance to β-lactams. In addition to this observation, PBP2a also localizes to multiple locations across the cell periphery, suggesting that it is also involved in peripheral cell wall maintenance in response to β-lactam treatment. Treatment with a synergistic combination of TXA707 and oxacillin, yields a unique phenotype in which PBP2a now forms discreet curved structures (Fig. 2.7J-M). This behavior differs from that observed upon treatment with vehicle, TXA707 alone, or oxacillin alone. We further explored this behavior using TEM. TEM micrographs of MRSA cells treated with a synergistic combination of oxacillin and TXA707 revealed a similar pattern to that observed in our immunofluorescence studies (Fig. 2.9). In these TEM micrographs, we observed thick and curved septal-like structures being formed in response to the combination treatment (Fig. 2.9D), structures that were absent in cells treated with either vehicle, oxacillin alone, or TXA707 alone (Figs. 2.9A-C).
Figure 2.1 Differential interference contrast (DIC) and fluorescence micrographs of MRSA LAC-F<sub>Ch</sub> cells treated for 3 hours with either DMSO vehicle (A-C) or 4 µg/mL (4x MIC) TXA707 (D-
F) just prior to visualization. Panels C and F depict enlargements of the regions enclosed by the
green and cyan boxes in panels B and E, respectively. The localization of FtsZ (red) is
schematically depicted in panel G, with the numbered arrows in the schematic depiction reflecting
the correspondingly numbered arrows in the fluorescence micrographs. The scale bars for panels
A-B and D-E represent 2 µm, while those for panels C and F represent 0.5 µm. The bar graph in
panel H shows the average diameter of the vehicle-treated cells \(n = 641\) as well as the TXA707-
treated cells \(n = 349\). The bar graph in panel I shows the prevalence (in %) of the various FtsZ
phenotypes observed in both vehicle- and TXA707-treated cells. Each percentage reflects an
average of 5 different fields of view, with the number of cells in each field of view ranging from
56 to 171. In both panels H and I, the indicated error bars reflect the standard deviation from the
mean. The statistical significance of differences in cell diameter and FtsZ phenotype 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 2.2 DIC and fluorescence micrographs of MRSA LAC-F<sub>ClP1</sub><sub>GFP</sub> cells treated for 3 hours with either DMSO vehicle (A-D) or 4 µg/mL (4x MIC) TXA707 (E-H) just prior to visualization.
The insets in panels B-D depict enlargements of the regions enclosed by the small cyan boxes. The localization of PBP1 (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, while those for the insets in panels B-D represent 0.5 µm. The bar graph in panel J shows the prevalence (in %) of the various FtsZ and PBP1 phenotypes observed in both vehicle-treated cells \((n = 919)\) and \(\text{TXA707}\)-treated cells \((n = 535)\). Each percentage reflects an average of 5 different fields of view, with the number of cells in each field of view ranging from 98 to 256. The indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in the FtsZ and PBP1 phenotypes were analyzed as described in the legend to Fig. 2.1.
Figure 2.3  DIC and fluorescence micrographs of MRSA LAC-FcBP3GFP cells treated as described in the legend to Fig. 2.2. The localization of PBP3 (green) and FtsZ (red) is schematically depicted.
in panel I, with the numbered arrows in the scheme reflecting the correspondingly numbered arrows in the fluorescence micrographs. The scale bars for panels A-H represent 2 µm. The bar graph in panel J shows the prevalence (in %) of the various FtsZ and PBP3 phenotypes observed in both vehicle-treated cells \((n = 545)\) and \textbf{TXA707}-treated cells \((n = 326)\). Each percentage reflects an average of 5 different fields of view, with the number of cells in each field of view ranging from 48 to 136. The indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in the FtsZ and PBP3 phenotypes were analyzed as described in the legend to Fig. 2.1.
Figure 2.4  DIC and fluorescence micrographs of MRSA LAC-FcBP2GFP cells treated as described in the legend to Fig. 2.2. 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 prevalence (in %) of the various FtsZ and PBP2 phenotypes observed in both vehicle-treated cells (n = 1,071) and TXA707-treated cells (n = 368). Each percentage reflects an average of 5 different fields of view, with the number of cells in each field of view ranging from 44 to 247. The indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in the FtsZ and PBP2 phenotypes were analyzed as described in the legend to Fig. 2.1.
Figure 2.5 DIC and fluorescence micrographs of MRSA LAC-FcrP4GFP cells treated as described in the legend to Fig. 2.2. The localization of PBP4 (green) and FtsZ (red) is schematically depicted.
in panel I, with the numbered arrows in the scheme reflecting the correspondingly numbered arrows in the fluorescence micrographs. The scale bars for panels A-H represent 2 μm. The bar graph in panel J shows the prevalence (in %) of the various FtsZ and PBP4 phenotypes observed in both vehicle-treated cells ($n = 300$) and TXA707-treated cells ($n = 326$). Each percentage reflects an average of 3 to 5 different fields of view, with the number of cells in each field of view ranging from 56 to 162. The indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in the FtsZ and PBP4 phenotypes were analyzed as described in the legend to Fig. 2.1.
Figure 2.6 DIC and fluorescence micrographs of MRSA LAC-Fch cells treated for 3 hours with either DMSO vehicle (A-D) or 4 µg/mL (4x MIC) TXA707 (panels E-H), followed by
immunostaining using a PBP2a-specific monoclonal mouse antibody and a goat anti-mouse Alexa Fluor® 488 secondary antibody prior visualization. The localization of PBP2a (green) and FtsZ (red) is schematically depicted in panel I, with the numbered arrows in the scheme reflecting the correspondingly numbered arrows in the fluorescence micrographs. The scale bars for panels A-H represent 2 µm. The bar graph in panel J shows the prevalence (in %) of the various FtsZ and PBP2a phenotypes observed in both vehicle-treated cells \((n = 536)\) and TXA707-treated cells \((n = 315)\). Each percentage reflects an average of 5 different fields of view, with the number of cells in each field of view ranging from 29 to 116. The indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in the FtsZ and PBP2a phenotypes were analyzed as described in the legend to Fig. 2.1. n.o. denotes none observed.
Figure 2.7  DIC and fluorescence micrographs of MRSA LAC-Fch cells treated for 3 hours with either 2 µg/mL (1/32x MIC) **oxacillin** (A-D), 0.5 µg/mL (1/2x MIC) **TXA707** (panels E-H), or a combination of 2 µg/mL **oxacillin** and 0.5 µg/mL **TXA707** (panels I-L), followed by
immunostaining of PBP2a as described in the legend to figure 2.6. The localization of PBP2a (green) and FtsZ (red) is schematically depicted in panel M, with the numbered arrows in the scheme reflecting the correspondingly numbered arrows in the fluorescence micrographs. The scale bars for panels A-L represent 2 µm.
Figure 2.8 Quantification of the cell diameter, FtsZ phenotype, and PBP2a phenotype results of the microscopy experiments depicted in Fig. 2.7. The bar graph in panel A shows the average diameter of the vehicle-treated cells \( (n = 535) \), oxacillin-treated cells \( (n = 835) \), TXA707-treated cells \( (n = 270) \), and the cells \( (n = 239) \) treated with a combination of both oxacillin and TXA707. The bar graph in panel B shows the prevalence (in %) of the various FtsZ and PBP2a phenotypes observed in the different treatment groups. Each percentage reflects an average of 5 to 6 different fields of view, with the number of cells in each field of view ranging from 28 to 325. In both panels A and B, the indicated error bars reflect the standard deviation from the mean. The statistical significance of differences in cell diameter, FtsZ phenotype, and PBP2a phenotype were analyzed as described in the legend to Fig. 2.1.
**Figure 2.9** Transmission electron micrographs of MRSA LAC-Fch cells treated for 3 hours with either vehicle (A), 0.5 µg/mL **TXA707** alone (B), 2 µg/mL **oxacillin** alone (C), or 0.5 µg/mL **TXA707** in combination with 2 µg/mL **oxacillin** (D). The scale bars for panels A-D represent 0.2 µm. The white arrows in panels A and C highlight septa in dividing cells, while the white arrows
in panels B and D highlight incomplete or aberrant septal structures. The yellow arrow in panel B highlights a bleb resulting from an aberrant attempt at division.
### Table 2.1 List of genetically modified MRSA LAC strains expressing fluorescence fusion forms of FtsZ and different PBPs

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
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<tbody>
<tr>
<td>LAC-F&lt;sub&gt;Ch&lt;/sub&gt;</td>
<td>LAC attB::[pLL39 Pspac-ftsZ-mCherry lacI Tet&lt;sup&gt;R&lt;/sup&gt;] pCM11-lacI Erm&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>LAC-F&lt;sub&gt;Ch&lt;/sub&gt;P&lt;sub&gt;1&lt;/sub&gt;GFP</td>
<td>LAC-F&lt;sub&gt;Ch&lt;/sub&gt; ΔpbpA::sfgfp-pbpA</td>
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<tr>
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<td>LAC-P2A&lt;sub&gt;Ch-1&lt;/sub&gt;</td>
<td>LAC ΔmecA::mecA-mCherry</td>
</tr>
<tr>
<td>LAC-P2A&lt;sub&gt;Ch-2&lt;/sub&gt;</td>
<td>LAC ΔmecA::mCherry-mecA</td>
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Erm = Erythromycin, Tet = Tetracycline
CHAPTER III
STRUCTURE-GUIDED DESIGN OF A FLUORESCENT PROBE FOR THE VISUALIZATION OF FTSZ IN CLINICALLY IMPORTANT GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIAL PATHOGENS


ABSTRACT

Addressing the growing problem of antibiotic resistance requires the development of new drugs with novel antibacterial targets. FtsZ has been identified as an appealing new target for antibacterial agents. Here, we describe the structure-guided design of a new fluorescent probe (BOFP) in which a BODIPY fluorophore has been conjugated to an oxazole-benzamide FtsZ inhibitor. Crystallographic studies have enabled us to identify the optimal position for tethering the fluorophore that facilitates the high-affinity FtsZ binding of BOFP. Fluorescence anisotropy studies demonstrate that BOFP binds the FtsZ proteins from the Gram-positive pathogens Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Streptococcus pyogenes, Streptococcus agalactiae, and Streptococcus pneumoniae with K_d values of 0.6-4.6 µM. Significantly, BOFP binds the FtsZ proteins from the Gram-negative pathogens Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii with an even higher affinity (K_d=0.2-0.8 µM). Fluorescence microscopy studies reveal that BOFP can effectively label FtsZ in all the above Gram-positive and Gram-negative pathogens. In addition, BOFP is effective at monitoring the impact of non-fluorescent inhibitors on FtsZ localization in these target pathogens. Viewed as a whole, our results highlight the utility of BOFP as a powerful tool for identifying new broad-spectrum FtsZ inhibitors and understanding their mechanisms of action.
INTRODUCTION

The discovery and development of antibiotics have saved millions of lives and revolutionized modern medicine(8). However, the alarming rise in multidrug-resistant (MDR) bacteria has threatened the usefulness of our current arsenal of antibiotics, leading the World Health Organization (WHO) to suggest an impending post-antibiotic era, where minor infections would become lethal(10). A recent WHO report highlights the global magnitude of an ever-worsening crisis, exacerbated by a chronic shortage of antibiotics capable of treating infections cause by MDR pathogens(2). Currently, the majority of drug candidates in the antibiotic pipeline are derivatives of known antibiotics that will provide only a short-term solution to the problem(2). Furthermore, many of these drug candidates have limited or no activity against MDR Gram-negative bacterial pathogens, which are particularly problematic to treat(2).

Addressing the global antibiotic resistance problem requires the development of new drug chemotypes and the identification of new antibacterial drug targets. The filamentous temperature-sensitive Z (FtsZ) protein has been identified as a promising new target for the development of novel antibiotics(11, 12, 16, 29-32, 34, 41, 80, 101). FtsZ has several properties that make it an appealing antibacterial drug target. It is an essential protein required for bacterial division(100, 102, 103). Inhibition of FtsZ has a bactericidal rather than bacteriostatic effect(32, 41), a property that reduces the potential for emergence of future resistance(104). FtsZ also has no functional human homolog, offering the potential to target this protein specifically and with minimal toxicity(31, 77). It is one of the most abundant and highly conserved cytoskeleton proteins among eubacteria(15), offering FtsZ inhibitors the potential for broad-spectrum antibacterial activity. Lastly, FtsZ is a “druggable” target whose function can be disrupted by small molecule targeting of a single site on the protein. While this latter property is appealing for a target protein, it also
introduces the potential for the development of resistance via single mutations (29, 31, 32, 34, 38, 39, 41, 101).

We have previously developed prodrugs of benzamide FtsZ inhibitors (PC190723 and TXA707) that are highly efficacious against infections caused by methicillin-resistant Staphylococcus aureus (MRSA) (30, 41, 80). One of these prodrugs (TXA709) is currently in phase I clinical trials (16). To date, the bulk of the compounds that have been validated as FtsZ inhibitors both in vitro with purified FtsZ and in bacterial cells are associated with potent activity against staphylococci, Mycobacterium tuberculosis, and select other Gram-positive bacterial strains, but weaker or no activity against Gram-negative species (13, 29, 31, 32, 35, 41). Demonstration of in vivo efficacy among these FtsZ inhibitors has been limited almost exclusively to the treatment of S. aureus and M. tuberculosis infections (28, 30-32, 34, 35, 41, 80, 101).

Advancing the development of new FtsZ inhibitors that can target a more expansive array of both Gram-positive and Gram-negative bacterial pathogens requires tools that allow us to screen for FtsZ inhibition in a broad range of bacterial species. Fluorescent antibiotics are useful tools for delineating the mechanisms underlying the antibacterial activities of compounds as well as the resistance phenotypes of bacteria (105). In addition, such tools can be used to screen for new antibiotic candidates with desired mechanisms of action (105). Early efforts aimed at developing fluorescent FtsZ inhibitors were centered on analogs of the benzamide inhibitor PC190723 (106). Several of these fluorescent analogs were shown to bind FtsZ from both S. aureus and Bacillus subtilis (SaFtsZ and BsFtsZ, respectively), though the interactions were weak (with estimated K_d values in the range of 11 to 29 µM for BsFtsZ at 25 °C), and none of the analogs were able to bind FtsZ from Escherichia coli (EcFtsZ) to a significant degree (106). One analog was used to visualize FtsZ in S. aureus and B. subtilis cells (106). However, visualization required prolonged (1- to 3-
hour) treatment with large concentrations of the analog (25 to 200 µM) and was lost upon pre-treatment with the parent inhibitor **PC190723**, limiting the usefulness of the analog as a screening tool for FtsZ inhibitors.

Here we report the structure-guided design and characterization of a next-generation fluorescent FtsZ probe (**BOFP**) that overcomes the limitations associated with the early-generation analogs. Our design incorporates an oxazole-benzamide FtsZ inhibitor (1)\(^{(29)}\) (shown in Fig. 3.1a), whose crystal structure in complex with SaFtsZ we have previously determined (PDB entry: 5XDU)\(^{(39)}\), conjugated to a boron-dipyrrromethene (BODIPY) fluorophore at the linker joining the oxazole and benzamide rings. Fluorescence anisotropy studies demonstrate that **BOFP** can target the FtsZ proteins from a broad range of Gram-positive pathogens (including *S. aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae*) with high affinity (K\(_d\) values in the range of 1.0 to 3.5 µM at 25 °C). Significantly, **BOFP** targets the FtsZ proteins from clinically important Gram-negative pathogens (including *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) with an even higher affinity (K\(_d\) values in the range of 0.3 to 0.6 µM at 25 °C). The crystal structure of **BOFP** in complex with SaFtsZ confirms that the probe targets the same site on the protein as the parent compound, while revealing contributions from the BODIPY functionality itself to the stability of the complex. Most importantly, fluorescence microscopy studies demonstrate that brief (5-minutes) exposure to **BOFP** (at a concentration of only 1.3 µM) can be used to visualize FtsZ in all the Gram-positive and Gram-negative bacterial pathogens listed above, even when pre-treated with other non-fluorescent FtsZ inhibitors. Taken together, our results indicate that **BOFP** can serve as a powerful tool for identifying new broad-spectrum FtsZ inhibitors and understanding their mechanisms of action.
MATERIALS AND METHODS

Bacterial strains and other reagents. *S. aureus* NRS705 (a USA100 MRSA strain isolated from the blood of a 14-day-old male in New York) was provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH. *E. coli* N43 *acrA* was a generous gift from Dr. Lynn Zechiedrich (Baylor College of Medicine, Houston, TX). MRSA LAC FtsZ-mCherry was generated as detailed in the Supplementary Information. All other bacterial strains were obtained from the American Type Culture Collection (ATCC). MRS, cation-adjusted Mueller-Hinton (CAMH), and tryptic soy media were from Becton-Dickinson. Todd Hewitt (TH) media was made from components specified by Becton-Dickinson. Lysed horse blood (LHB) was from Hardy Diagnostics, the sodium salt of 2'-deoxyguanosine-5'-(α,β)-methylene-triphosphate (GMPCPP) was from Jena Bioscience, and phosphate-buffered saline (PBS) was from Lonza. Pentamidine isethionate, isopropyl β-d-1-thiogalactopyranoside (IPTG), and PC190723 were from Sigma. 3-(5,5-difluoro-7,9-dimethyl-5H-5λ4,6λ4-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoic acid (BODIPY FL-COOH) was from Lumiprobe Corp.

Compound synthesis. 1, 2, 3, and TXA707, were synthesized as previously described\(^2\). BOFP was synthesized as detailed in the Supplementary Information.

Crystallization and structure determination of SaFtsZ in complex with 2 and BOFP. SaFtsZ\(_{12-316}\) was cloned, expressed, and purified as described previously\(^3\). After SaFtsZ\(_{12-316}\) was crystallized by the sitting-drop vapor-diffusion technique at 20 °C in reservoir conditions based on JBScreen pentaerythritol 2 (Jena Bioscience), racemic mixtures of the R and S enantiomeric forms of 2 or BOFP were introduced by soaking. For the complex of 2 with SaFtsZ, the protein was
crystallized at 9.5 mg/mL under conditions of 100 mM Tris-HCl (pH 7.8), 45% (w/v) pentaerythritol propoxylate 629 (PEP629), and 300 mM KCl. After 3 weeks, the crystal was soaked in the same reservoir supplemented with 5 mM 2 for 3 days. For the BOFP complex, the protein was crystallized at 20 mg/mL under conditions of 100 mM HEPES (pH 8.0) and 39% (w/v) PEP629. After 7 days, the crystal was soaked in the same reservoir supplemented with 1.4 mM BOFP for 3 days.

The crystal of the 2-SaFtsZ complex was flash-frozen in a nitrogen gas stream at -180 °C without cryoprotectants. The crystal of the BOFP-SaFtsZ complex was briefly soaked in a cryoprotectant solution containing 100 mM HEPES (pH 8.0), 39% (w/v) PEP629, and 25% (v/v) glycerol and then flash-frozen in the same manner as described above. X-ray diffraction data from the crystals of the 2-SaFtsZ and BOFP-SaFtsZ complexes were collected at SPring-8 BL44XU (Hyogo, Japan) under a cryogenic nitrogen gas stream at 100 K. Diffraction data were processed and scaled with HKL2000(107) and XDS(108), respectively. The phases for both complexes were determined by molecular replacement with Phaser in the CCP4 suite(109) using the previously determined structure of the SaFtsZ-GDP complex (PDB entry: 3VOA) (110) as a search model. Both models were refined with Refmac5(111) and PHENIX(112), with manual modification using COOT(113). The refined structures were validated with MolProbity(114). Data collection and refinement statistics are summarized in Table 3.2. The final atomic coordinates and structure factor amplitudes have been deposited in the RCSB Protein Data Bank (PDB entries: 6KVP and 6KVQ). Figures were prepared with PyMOL (Schrödinger).

**Fluorescence anisotropy assays for the binding of BOFP to FtsZ proteins.** Fluorescence anisotropy experiments were performed using an AVIV model ATF105 spectrofluorometer at 15, 25, or 37 °C. In these experiments, bandwidths were set to 4 nm in both the excitation and emission
directions, with the excitation and emission wavelengths being set at 488 nm and 510 nm, respectively. **BOFP** (0.1 µM) was titrated with increasing concentrations (ranging from 0 to 12 µM) of FtsZ in 120 µL of buffer containing 50 mM Tris-HCl (pH 7.6) and 50 mM KCl. After each protein addition, the samples were equilibrated for 3 minutes, whereupon the fluorescence anisotropy was measured.

Plots of the fluorescence anisotropy (r) of **BOFP** as a function of FtsZ concentration (as shown in Figs. 3.2, 3.3, and 3.5) were analyzed by non-linear least squares regression using the following 1:1 binding formalism:

\[
r = r_0 + \frac{r_\infty - r_0}{2[C]_{tot}} \left( ([C]_{tot} + [P]_{tot} + K_d) - \sqrt{([C]_{tot} + [P]_{tot} + K_d)^2 - 4[C]_{tot}[P]_{tot}} \right) -(Eq. 3.1)
\]

In this equation, \(r_0\) is the anisotropy of the protein-free compound, \(r_\infty\) is the anisotropy of the compound in the presence of an infinite concentration of FtsZ, \([C]_{tot}\) is the total concentration of the compound, and \([P]_{tot}\) is the total concentration of protein with each addition. These analyses yielded the equilibrium dissociation constant (\(K_d\)) for each binding reaction.

The binding free energy (\(\Delta G\)) at temperature \(T\) was derived from the corresponding \(K_d\) value determined at \(T\) using the following relationship:

\[
\Delta G = -RT \ln \left( \frac{1}{K_d} \right) \quad (Eq. 3.2)
\]

The binding enthalpy (\(\Delta H\)) and entropy (\(\Delta S\)) were derived from linear fits of the \(\ln(1/ K_d)\) vs. \(1/T\) plots shown in Figs. 3.3f and 5e using the following relationship:

\[
\ln \left( \frac{1}{K_d} \right) = -\frac{\Delta H}{R} \left( \frac{1}{T} \right) + \frac{\Delta S}{R} \quad (Eq. 3.3)
\]

The impact of guanosine nucleotide and magnesium on the binding of **BOFP** to SaFtsZ was assessed at 37 °C in the same buffer described above. In these studies, the anisotropy of 0.1
µM BOFP alone or in the presence of 10 µM SaFtsZ was measured, with the latter also being measured in the presence of 0.1 mM GMPCPP (a non-hydrolyzable GTP analog), 10 mM MgCl₂, or both.

In comparative control experiments, the anisotropy of 0.1 µM BODIPY FL-COOH or BOFP alone or in the presence of SaFtsZ (10 µM), SpyFtsZ (10 µM), KpFtsZ (2 µM), or PaFtsZ (2 µM) was measured at 37 °C in the same buffer described above.

A quartz ultra-micro cell (Hellma) with a 2 × 5 mm aperture and a 15 mm center height was used for all measurements. The pathlengths in the excitation and emissions directions were 1 and 0.2 cm, respectively. All steady-state anisotropy experiments were conducted in at least triplicate, with the reported anisotropies reflecting the average values.

Minimum Inhibitory Concentration (MIC) Assays. MIC assays of 1, 3, and BOFP were conducted by standard broth microdilution in TH media. Briefly, log-phase S. aureus NRS705 (MRSA) cells were added to 96-well microtiter plates (at 5 x 10⁵ CFU/mL) containing 2-fold serial dilutions of each test compound in 0.1 mL of TH broth, with each compound concentration being present in duplicate. The MIC is defined as the lowest compound concentration at which growth is ≥90% inhibited after 18-24 hours of aerobic growth.

Differential interference contrast and fluorescence microscopy. All differential interference contrast (DIC) and fluorescence microscopy experiments were conducted using an Olympus BX50 microscope equipped with an X-cite Exacte 200W mercury lamp, a 100× Olympus UPLSAPO oil immersion objective (1.40 aperture), as well as both Chroma ET-EGFP (FITC/Cy2) and ET mCherry, Texas Red filters. Images were captured using a QImaging Retiga R3 charge-coupled device (CCD) camera and the Ocular-Version 2.0 software package (QImaging).
For visualizing FtsZ in Gram-positive bacteria using **BOFP**, the bacterial cells were grown to log-phase in media suitable for each individual pathogen. Specifically, *S. aureus* NRS705 (MRSA) was grown in tryptic soy broth (TSB), *E. faecalis* ATCC 29212 and *E. faecium* ATCC 19434 were grown in lactobacilli MRS broth, *S. agalactiae* ATCC 12386 and *S. pneumoniae* ATCC 49619 were grown in TH broth, and *S. pyogenes* ATCC 19615 was grown in CAMH broth supplemented with 3% (v/v) LHB. For each Gram-positive bacterial strain, a total of 1 mL of cell culture was centrifuged at 15,000 × g for 1 minute and washed 2-3 times with 1 mL of PBS. After the final wash, the pelleted cells were resuspended in 500 µL of PBS containing 1 µg/mL of **BOFP** and incubated in the dark for 5 minutes at room temperature. The cells were then centrifuged at 15,000 × g for 1 minute, washed twice with 1 mL of PBS, and subsequently resuspended in 200 µL of PBS. 8 µL of this final cell suspension was then spread on a 0.25 mm layer of 1.5% high-resolution agarose (Sigma) in PBS, which was mounted on a standard 75 x 25x 1 mm microscope slide (Azer Scientific) using a 1.7 x 2.8 x 0.025 cm Gene Frame (ThermoFisher). A 24 x 40 mm cover slip (Azer Scientific) was then applied to the agarose pad to prepare the slide for microscopic visualization. Comparative control experiments with 1 µg/mL **BODIPY FL-COOH** were conducted in *S. aureus* NRS705 cells as described above for **BOFP**.

For visualizing FtsZ in Gram-negative bacteria using **BOFP**, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 27853, and *A. baumannii* ATCC 19606 were grown to log-phase in CAMH broth. For each Gram-negative bacterial strain, a total of 1 mL of cell culture was centrifuged at 15,000 × g for 1 minute and washed twice with 1 mL of Tris-buffered saline (TBS) composed of 50 mM Tris-HCl pH 7.6 and 150 mM NaCl. After the final wash, the pelleted cells were resuspended in 500 µL of TBS containing 1 µg/mL of **BOFP** and pentamidine isethionate (at 0.875 mg/mL for *E. coli* and 3.5 mg/mL for the other three strains).
The resuspended cells were then incubated in the dark for 5 minutes at room temperature, centrifuged at 15,000 × g for 1 minute, washed twice with 1 mL of TBS, and subsequently resuspended in 200 µL of TBS. This final cell suspension was then prepared for microscopy as described for the Gram-positive bacterial strains. Comparative control experiments with 1 µg/mL BODIPY FL-COOH were conducted in *K. pneumoniae* ATCC 13883 cells as described above for BOFP.

To visualize the impact of treatment with the FtsZ inhibitor 1 using BOFP, *S. aureus* NRS705 (MRSA), *E. coli* N43, and *K. pneumoniae* ATCC 10031 were grown to log-phase in CAMH broth and diluted to an OD<sub>600</sub> of 0.1. Each cell culture was then treated with either DMSO vehicle or 1 at 4× MIC (1 µg/mL for *S. aureus* or 4 µg/mL for *E. coli* and *K. pneumoniae*) for 3 hours at 37 °C. Following this treatment, 1-5 mL of each culture was centrifuged at 15,000 × g for 1 minute and washed twice with 1 mL of PBS (for *S. aureus*) or TBS (for *E. coli* and *K. pneumoniae*). The resulting *S. aureus* cell pellets were further processed and labeled with BOFP as described above for the Gram-positive bacterial strains and the resulting *E. coli* and *K. pneumoniae* cell pellets were further processed and labeled with BOFP as described above for the Gram-negative bacterial strains. The impact of treating *S. aureus* NRS705 cells with the FtsZ inhibitors PC190723 and TXA707 at 4× MIC (2 µg/mL for PC190723 and 4 µg/mL for TXA707) was also examined using BOFP as described above.

To visualize the impact of treating MRSA LAC FtsZ-mCherry with 1, cells were grown to log-phase in CAMH broth and diluted to an OD<sub>600</sub> of 0.1. The cells were then treated with 10 µM IPTG and either DMSO vehicle or 1 at 4× MIC (0.25 µg/mL for PC190723 and 4 µg/mL for TXA707) for 3 hours at 37 °C. Following this treatment, 1-5 mL of each culture was centrifuged at 15,000 × g for 1 minute and washed twice
with 1 mL of PBS. The resulting cell pellets were further processed and labeled with BOFP as described above for the Gram-positive bacterial strains.
RESULTS AND DISCUSSION

Structure-guided identification of a suitable site on the oxazole-benzamide FtsZ inhibitor for conjugation of a fluorophore. A clue to a potential site on the oxazole-benzamide FtsZ inhibitor 1 for conjugation of a fluorophore came from the crystal structure we determined for the complex of SaFtsZ\textsubscript{12–316} (the enzymatic domain of SaFtsZ, residues 12–316) with 2\textsuperscript{(29)}, a methyl analog of 1 whose chemical structure is shown in Fig. 3.1a. The only difference between the two compounds is the presence of a methyl group (shown in red in Fig. 3.1a) on the linker connecting the oxazole and difluorobenzamide rings of 2 that is absent in 1. This difference makes 2 a chiral molecule, while 1 is achiral. A racemic mixture of the R and S enantiomeric forms of 2 was dissolved in DMSO and introduced into crystals of SaFtsZ\textsubscript{12–316} by soaking. The structure of the R enantiomer of 2 [(R)-2] in complex with SaFtsZ\textsubscript{12–316} was determined at 1.4 Å resolution. An extra electron density was clearly observed in the cleft between \textit{N}- and \textit{C}-terminus domain, which enabled us to determine the position and orientation of (R)-2 with an occupancy of 1.0 (Fig. 3.1b). We have previously reported the structure of 1 in complex with SaFtsZ\textsubscript{12–316}\textsuperscript{(39)}. (R)-2 binds the same protein cleft as 1 in a similar orientation to that observed in the structure of the 1-SaFtsZ\textsubscript{12–316} complex (see the overlay of 1 and (R)-2 depicted in Fig. 3.1c). Note that no SaFtsZ complexes were observed with the S enantiomer of 2, suggesting that SaFtsZ is selective for the R over the S enantiomer.

Inspection of the crystal structure of (R)-2 in complex with SaFtsZ\textsubscript{12–316} reveals that the methyl group distinguishing (R)-2 from 1 does not engage in significant interactions with SaFtsZ, but rather is oriented away from the cleft of the FtsZ molecule (as highlight by the red arrows in Fig. 3.1b,c). This observation suggested to us that a bulky fluorescent moiety could be conjugated
to the same site on the linker in 1 as the methyl group in 2 without disrupting the FtsZ binding interaction to a significant degree.

**Design of a BODIPY-conjugated fluorescent derivative of 1 (BOFP) that binds SaFtsZ with affinity in the K_d range of 0.9 to 3.1 µM.** Armed with the structural results described above, we designed and synthesized BOFP, a fluorescent analog of 1 in which a BODIPY fluorophore is conjugated to the desired site on 1 by reaction of BODIPY FL carboxylate (BODIPY FL-COOH) with 3(29, 31) (a hydroxymethyl analog of 1 prepared as a racemic mixture of R and S enantiomers) as schematically depicted in Fig. 3.2a. This reaction resulted in a racemic mixture of BOFP as well. In our initial characterizations, we sought to determine whether BOFP could still bind SaFtsZ, as hypothesized in our structure-guided design approach. Toward this end, we used fluorescence anisotropy to monitor the interaction of BOFP with SaFtsZ at three different temperatures (15, 25, and 37 °C). Significantly, the fluorescence anisotropy (r) of BOFP increases markedly with added SaFtsZ (Fig. 3.2b), indicating the presence of a binding interaction. No such anisotropy change was observed in control studies with unreacted BODIPY FL-COOH (Fig. 3.S1). This important control observation not only demonstrates that BODIPY FL-COOH does not interact with the target FtsZ protein but also that the ester linkage connecting the BODIPY fluorophore in the BOFP conjugate was not hydrolyzed during the binding reactions, as BODIPY FL-COOH would be the fluorescent product of such a hydrolysis.

Analysis of the r-based binding isotherms of BOFP with the 1:1 binding formalism embodied by Eq. 3.1 yielded excellent fits of the experimental data points (depicted by the solid curves in Fig. 3.2b) and the corresponding K_d values listed in Table 3.1, which ranged from 0.88 ± 0.08 µM at 15 °C to 3.14 ± 0.13 µM at 37 °C. These gratifying results indicate that BOFP can bind SaFtsZ with a robust affinity in the sub- to low-micromolar K_d range.
The binding of BOFP to SaFtsZ does not require the presence of GTP or magnesium. The filamentation of SaFtsZ requires the presence of both GTP and magnesium (115). Note that neither of these reagents was present in the fluorescence anisotropy binding studies depicted in Fig. 3.2b, indicating that the binding of BOFP to SaFtsZ does not require the presence of GTP or magnesium. This observation markedly contrasts the fluorescence anisotropy studies previously reported by Artola et al. (106), which demonstrated that the fluorescent analogs of the benzamide FtsZ inhibitor PC190723 required both GTP and magnesium in order to bind SaFtsZ. For comparative purposes, we sought to determine whether the presence of a non-hydrolyzable analog of GTP (GMPCPP) and magnesium exerted an impact on the binding of BOFP to SaFtsZ, as reflected by a change in fluorescence anisotropy. At identical concentrations of BOFP (0.1 µM) and SaFtsZ (10 µM), the presence of neither GMPCPP alone (at 0.1 mM) nor MgCl₂ alone (at 10 mM) has a significant effect on the anisotropy of SaFtsZ-bound BOFP (Fig. 3.S2), confirming that the binding of BOFP to SaFtsZ is independent of either GTP or magnesium. The presence of both GMPCPP and MgCl₂ results in a modest increase in the anisotropy of bound BOFP (Fig. 3.S2), which likely reflects the filamentation of BOFP-bound SaFtsZ induced by the combination of GMPCPP and magnesium.

BOFP binds in the same cleft of SaFtsZ as 1 and 2. We next sought to determine the crystal structure of BOFP in complex with SaFtsZ₁₂₋₃₁₆. Toward this end, a racemic mixture of the R and S enantiomeric forms of BOFP in DMSO was introduced into crystals of SaFtsZ₁₂₋₃₁₆ by soaking. The structure of the R enantiomer of BOFP [(R)-BOFP] in complex with SaFtsZ₁₂₋₃₁₆ was determined at 1.6 Å resolution. Although the electron density of (R)-BOFP was not quite as robust as that of (R)-2 due to a lower occupancy of 0.6, we were able to confirm that (R)-BOFP does indeed bind in the same cleft (compare Figs. 3.1b and 3.2c). The conjugated BODIPY extends toward the outside of the FtsZ molecule, away from the binding cleft. As hypothesized,
interactions between the 1 portion of (R)-BOFP and SaFtsZ are similar to those exhibited by 1 (see the overlay of 1 and (R)-BOFP depicted in Fig. 3.2d). In addition to these interactions, the complex of (R)-BOFP with SaFtsZ is further stabilized by hydrophobic interactions between the BODIPY moiety and the hydrophobic surface formed by residues Ile228, Val230, and Val307 (Fig. 3.2e), with these interactions being unlikely to hamper the ability of SaFtsZ to self-associate into filaments (Fig. 3.S3).

As seen with 2, no SaFtsZ complexes were observed with the S enantiomer of BOFP, further indicative of the selectivity of SaFtsZ for the R versus the S enantiomeric form. Previous studies by Stokes et al. have shown that the R enantiomeric form of 3 has significantly greater activity against methicillin-sensitive S. aureus (MSSA) than the S enantiomeric form(29). This enhanced antistaphylococcal activity of the R enantiomer likely reflects the corresponding selectivity of SaFtsZ for the R enantiomeric form.

Note that the FtsZ targeting of BOFP confers the compound with antistaphylococcal activity, though this activity is somewhat reduced relative to the parent compounds 1 and 3 (MIC versus MRSA NRS705 = 0.25, 0.5, and 1.0 µg/mL for 1, 3, and BOFP, respectively). The reduced activities of both 3 and BOFP relative to 1 may be due in part to 3 and BOFP being racemic mixtures of active R and weakly active S enantiomers. In the aggregate, our collective fluorescence anisotropy, crystallographic, and antibacterial results for BOFP serve to validate our structure-guided design approach.

**BOFP can target the FtsZ proteins from a broad range of clinically important Gram-positive bacterial pathogens, including enterococcal and streptococcal species.** In addition to SaFtsZ, we also sought to determine whether BOFP can target the FtsZ proteins from other Gram-positive bacterial pathogens, including E. faecalis (EfsFtsZ) E. faecium (EfMftsZ), S. pyogenes (SpyFtsZ),
S. agalactiae (SagFtsZ), and S. pneumoniae (SpnFtsZ). Fluorescence anisotropy studies conducted at 15, 25, and 37 °C reveal that addition of each of the five target FtsZ proteins increases the anisotropy of **BOFP** significantly (Fig. 3.3a-e), indicative of a binding interaction between the probe and each of the host proteins. Significantly, no such binding interactions were observable with **BODIPY FL-COOH** (as exemplified by the SpyFtsZ results shown in Fig. 3.S1). Thus, **BOFP** can target not only SaFtsZ, but also the FtsZ proteins from a broad range of other clinically important Gram-positive pathogens.

Analysis of the anisotropy isotherms in Fig. 3.3 with Eq. 3.1 yielded outstanding fits of the experimental data points (as depicted by the solid curves), with the $K_d$ values derived from these fits being listed in Table 3.1. $K_d$ values for **BOFP** binding to the Gram-positive FtsZ proteins ranged from $1.72 \pm 0.06 \mu M$ at 15 °C to $4.62 \pm 0.12 \mu M$ at 37 °C for EfsFtsZ, $2.50 \pm 0.14 \mu M$ at 15 °C to $3.14 \pm 0.22 \mu M$ at 37 °C for EfmFtsZ, $0.91 \pm 0.06 \mu M$ at 15 °C to $1.55 \pm 0.08 \mu M$ at 37 °C for SpyFtsZ, $0.62 \pm 0.05 \mu M$ at 15 °C to $1.31 \pm 0.04 \mu M$ at 37 °C for SagFtsZ, and $3.02 \pm 0.30 \mu M$ at 15 °C to $3.81 \pm 0.69 \mu M$ at 37 °C for SpnFtsZ. These $K_d$ ranges are similar in magnitude to that observed for SaFtsZ. Recall that Artola et al. demonstrated the binding of their early-generation fluorescent FtsZ inhibitors only to SaFtsZ and BsFtsZ, while reporting $K_d$ values in the range of 11 to 29 µM for BsFtsZ at 25 °C(106). Significantly, the lower range of $K_d$ values we observe for the binding of **BOFP** to all six Gram-positive FtsZ proteins at 25 °C (1.02 ± 0.09 to 3.49 ± 0.25 µM) indicates a much broader spectrum of FtsZ targeting as well as a binding affinity that is approximately 3- to 29-fold higher.

**Brief exposure to a low concentration of BOFP effectively labels FtsZ in live S. aureus, E. faecalis, E. faecium, S. pyogenes, S. agalactiae, and S. pneumoniae cells.** Having demonstrated the high-affinity binding of **BOFP** to the Gram-positive FtsZ proteins SaFtsZ, EfsFtsZ, EfmFtsZ,
SpyFtsZ, SagFtsZ, and SpnFtsZ, we explored the potential of the probe to label FtsZ in live cells of the corresponding pathogens themselves. Fig. 3.4 shows differential interference contrast (DIC) and fluorescence micrographs of *S. aureus*, *E. faecalis*, *E. faecium*, *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* cells labeled for 5 minutes with 1 µg/mL (1.3 µM) **BOFP**. In each of the six pathogens, bright bands of fluorescence staining are clearly visible at midcell (as highlighted by the arrows in Fig. 3.4b,d,f,h,j,l), consistent with the labeling of FtsZ Z-rings. Additional fluorescence staining (though weaker than that at midcell) is also evident along the periphery of each cell, suggesting that FtsZ is also localized throughout the cell membrane. These results indicate that brief exposure to a low concentration of **BOFP** affords outstanding visualization of FtsZ and its localization patterns in live Gram-positive bacterial cells.

To ensure that the ester linkage connecting the BODIPY fluorophore in the **BOFP** conjugate was not being hydrolyzed by the bacterial cells, we examined the potential, if any, of **BODIPY FL-COOH** to label FtsZ in *S. aureus* cells. Significantly, no fluorescence staining of the *S. aureus* cells was detectable upon treatment with 1 µg/mL **BODIPY FL-COOH** for 5 minutes. This behavior markedly contrasts the clear staining patterns observed when the cells are treated similarly with **BOFP** (Fig. 3.4S4). These results confirm that **BOFP** is not hydrolyzed by the cells during the treatment regimen and that the staining patterns observed were not the result of nonspecific interactions with a fluorescent product of hydrolysis.

**BOFP targets FtsZ proteins from Gram-negative bacterial pathogens with an even higher affinity than FtsZ proteins from Gram-positive pathogens.** In addition to targeting Gram-positive FtsZ proteins, we sought to determine whether **BOFP** could also target Gram-negative FtsZ proteins. To this end, we used fluorescence anisotropy to explore the interactions of **BOFP** with the FtsZ proteins from the four Gram-negative pathogens, *E. coli* (EcFtsZ), *K. pneumoniae*
(KpFtsZ), *P. aeruginosa* (PaFtsZ), and *A. baumannii* (AbFtsZ), with the resulting anisotropy profiles acquired at 15, 25, and 37 °C being depicted in Fig. 3.5a-d. Inspection and analysis of these anisotropy profiles reveals that **BOFP** binds to all four Gram-negative FtsZ proteins with sub-micromolar affinity. At 25 °C, the $K_d$ values for EcFtsZ, KpFtsZ, PaFtsZ, and AbFtsZ are $0.28 \pm 0.02$, $0.58 \pm 0.04$, $0.36 \pm 0.06$, and $0.55 \pm 0.02 \mu M$, respectively (Table 3.1). A comparison of the $K_d$ values at 25 °C listed in Table 3.1 indicates that **BOFP** binds the Gram-negative FtsZ proteins with an approximately 2- to 12-fold higher affinity than the Gram-positive FtsZ proteins. Thus, in striking contrast to the early-generation fluorescent FtsZ inhibitors reported by Artola *et al.* (106), **BOFP** can target a broad range Gram-negative FtsZ proteins with a high degree of affinity. As observed with the Gram-positive FtsZ proteins, no binding interactions with **BODIPY FL-COOH** were detectable with the Gram-negative FtsZ proteins (as exemplified by the KpFtsZ and PaFtsZ results shown in Fig. 3.S1).

**For the majority of target FtsZ proteins studied, enthalpy provides a significant driving force for the binding of BOFP.** We used the temperature dependence of the $K_d$ values for the binding of **BOFP** to the Gram-positive and Gram-negative FtsZ proteins to derive the thermodynamic parameters associated with the binding reactions. Free energy changes ($\Delta G$) at 37 °C (310 K) were derived from the corresponding $K_d$ values using Eq. 3.2, while enthalpy and entropy changes ($\Delta H$ and $\Delta S$, respectively) were determined from linear fits of the $\ln(1/ K_d)$ vs. $1/T$ plots shown in Figs. 3.3f and 3.5e with Eq. 3.3. The resulting thermodynamic parameters are listed in Table 3.1. For seven of the ten FtsZ proteins studied (SaFtsZ, EfsFtsZ, SpyFtsZ, SagFtsZ, EcFtsZ, KpFtsZ, and PaFtsZ), $\Delta H$ contributes >50% to the observed $\Delta G$ of binding, with the enthalpic contribution to binding being 100% for two of those seven FtsZ proteins (SaFtsZ and EfsFtsZ). As suggested by our crystal structure of **BOFP** in complex with SaFtsZ, these favorable enthalpic contributions
to binding likely stem from the extensive array of favorable van der Waals contacts between the host protein and both the I and BODIPY portions of the probe (Fig. 3.2c-e). For the remaining three FtsZ proteins (EfmFtsZ, SpnFtsZ, and AbFtsZ), $\Delta S$ contributes >50% to the observed $\Delta G$ of binding. These favorable entropic contributions to binding may reflect favorable binding-induced changes in hydration and/or conformation of the host proteins.

In addition to Gram-positive bacterial cells, BOFP also labels FtsZ effectively in live Gram-negative cells of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. Armed with knowledge that BOFP binds to the Gram-negative FtsZ proteins EcFtsZ, KpFtsZ, PaFtsZ, and AbFtsZ with an even higher affinity than any of the Gram-positive FtsZ proteins, we further explored the potential of the probe to label FtsZ in live Gram-negative bacterial cells. When exposed to BOFP in a similar manner (1 µg/mL for 5 minutes) to that described above for the Gram-positive bacteria, little or no FtsZ labeling in the Gram-negative cells was observable by fluorescence microscopy (as exemplified by the *K. pneumoniae* results shown in Fig. 3.S5b). Previous studies have indicated that the large size of fluorescent antibiotics resulting from the conjugation of bulky fluorophores restricts the passage of the agents across the outer membrane of Gram-negative cells(116). Stokes et al. have shown that pentamidine can effectively permeabilize the outer membrane of Gram-negative bacterial cells to large antibiotics that would normally be unable to cross the membrane(117). When co-treating *K. pneumoniae* cells with 1 µg/mL BOFP and 3.5 mg/mL pentamidine isethionate for 5 minutes, bright fluorescence staining becomes visible both at midcell and along the cell periphery (compare Fig. 3.S5b and d). Co-treatment of *E. coli*, *P. aeruginosa*, and *A. baumannii* cells results in a similar fluorescence staining pattern to that observed in *K. pneumoniae* cells (Fig. 3.6b,d,f,h). The fluorescent bands visible at midcell are consistent with the labeling of FtsZ Z-rings (highlighted by the arrows in Figs. 3.S5d and
6b,d,f,h), with the peripheral staining reflecting the presence of FtsZ in the cell membrane as well. Note that no such FtsZ staining was detectable in *K. pneumoniae* cells co-treated for 5 minutes with 1 µg/mL **BODIPY FL-COOH** and 3.5 mg/mL pentamidine isethionate (Fig. 3.S6), thus confirming that **BOFP** is not being hydrolyzed by the Gram-negative cells during the labeling procedure. Viewed as a whole, our results indicate that **BOFP** is useful for visualizing FtsZ not only in live Gram-positive cells, but also in live Gram-negative cells.

**BOFP can also be used to visualize the impact of non-fluorescent FtsZ inhibitors on the localization of FtsZ in both Gram-positive and Gram-negative bacterial cells.** For **BOFP** to be useful as a tool for identifying new FtsZ inhibitors in a live cell-based assay, it must facilitate the detection of changes in FtsZ localization induced by non-fluorescent test compounds with the potential for FtsZ inhibition. Toward this end, we tested the ability of **BOFP** to visualize the impact of the oxazole-benzamide FtsZ inhibitor 1 on FtsZ localization in live *S. aureus*, *E. coli*, and *K. pneumoniae* cells, with the results being shown in Fig. 3.7. As expected with a known FtsZ inhibitor, treatment with 1 (at 4x MIC for 3 hours) induces a significant change in cell morphology consistent with the impairment of cell division (compare Fig. 3.7a,c,e with Fig. 3.7g,i,k). This morphological change takes the form of cell enlargement in cocci like *S. aureus* and filamentation in rods like *E. coli* and *K. pneumoniae*. Significantly, **BOFP** effectively labels FtsZ in the cells treated with 1, showing clear mislocalization of FtsZ and an absence of Z-rings in any of the treated cells (Fig. 3.7h,j,l). In addition, the presence of FtsZ in the membranes of cells treated with 1 is reduced relative to that in the membranes of untreated cells. In *S. aureus*, we observed similar results for cells treated with the benzamide FtsZ inhibitors **PC190723** and **TXA707** (Fig. 3.S7).

We sought to verify that the **BOFP** staining observed in *S. aureus* cells treated with non-fluorescent FtsZ inhibitors still reflected specific interaction with FtsZ and not off-target
interactions. To this end, we used **BOFP** to monitor the impact of treatment with 1 on FtsZ localization in live MRSA LAC cells that expresses a FtsZ-mCherry fusion protein (MRSA LAC FtsZ-mCherry). As shown in Fig. 3.8, the green fluorescence associated with **BOFP** labeling colocalizes with the red fluorescence associated with the induced expression of FtsZ-mCherry in both vehicle-treated and 1-treated cells. Thus, the observed **BOFP** labeling reflects specific interactions with FtsZ even in cells treated with non-fluorescent FtsZ inhibitors.

Our collective results highlight the usefulness of **BOFP** as a screening tool for identifying novel FtsZ inhibitors in both Gram-positive and Gram-negative pathogens. The fluorescent FtsZ inhibitors previously reported by Artola *et al.* lacked this utility, as their ability to label FtsZ was significantly diminished in live cells treated with known FtsZ inhibitors like **PC190723**(106).
CONCLUSIONS

This work describes the structure-guided design of a fluorescent FtsZ-targeting probe (BOFP) consisting of an oxazole-benzamide FtsZ inhibitor conjugated to a BODIPY fluorophore. Crystallographic studies demonstrate that BOFP targets the same FtsZ site as inhibitors like PC190723 and TXA707, while also highlighting favorable contributions from the conjugated BODIPY moiety itself to the FtsZ binding reaction. Fluorescence anisotropy studies with BOFP provide the first demonstration of a fluorescent probe capable of targeting the FtsZ proteins from a broad range of clinically relevant Gram-positive and Gram-negative bacterial pathogens with a high degree of affinity. In addition, fluorescence microscopy studies highlight the utility of BOFP for visualizing FtsZ in these pathogenic bacteria as well as for visualizing the impact of non-fluorescent inhibitors on FtsZ localization. These properties make BOFP a robust tool for identifying new broad-spectrum FtsZ inhibitors and understanding their mechanisms of action.
Figure 3.1  (a) Chemical structures of the oxazole-benzamide FtsZ inhibitors 1 and 2. The methyl group in 2 that differentiates this compound from 1 is highlighted in red. 2 was prepared as a racemic mixture of R and S enantiomers. (b) Expanded view of the binding site for the R enantiomer of 2 [(R)-2] in complex with SaFtsZ, with the $F_0 - F_c$ omit map (cyan) being contoured at 3.0$\sigma$. The anomalous difference map (purple) is contoured at 4.0$\sigma$. (c) Superposition of the SaFtsZ–(R)-2 complex (orange) with the corresponding SaFtsZ–1 complex (blue). The methyl group in 2 shown in red in (a) is highlighted by the red arrows in (b) and (c).
Figure 3.2  (a) Scheme for the synthesis of BOFP by reacting 3 with BODIPY FL-COOH in
CH2Cl2 containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP). Both 3 and BOFP were prepared as racemic mixtures of the R and S enantiomers. The tethered hydroxymethyl functionality on 3 and BODIPY functionality on BOFP are both highlighted in red. (b) Fluorescence anisotropy profiles of 0.1 µM BOFP as a function of increasing concentrations of SaFtsZ. The titration experiments were conducted at 15 °C (red), 25 °C (black), or 37 °C (cyan) in solution containing 50 mM Tris-HCl (pH 7.6) and 50 mM KCl. The solid lines reflect non-linear least squares fits of the experimental data points with Eq. 3.1. (c) Expanded view of the binding site for the R enantiomer of BOFP [(R)-BOFP] in complex with SaFtsZ, with the F0 – Fc omit map (cyan) being contoured at 2.0σ. (d) Superposition of the SaFtsZ–(R)-BOFP complex (green) with the corresponding SaFtsZ–1 complex (blue). (e) Hydrophobic interactions at the surface interface between the BODIPY moiety of BOFP and residues Ile228, Val230, and Val307 of SaFtsZ.
Figure 3.3  Fluorescence anisotropy profiles of 0.1 µM BOFP as a function of increasing concentrations of EfsFtsZ (a), EfmFtsZ (b), SpyFtsZ (c), SagFtsZ (d), or SpnFtsZ (e). Acquisition and display parameters are as described in the legend to Figure 3.2b. Panel (f) shows plots of ln(1/K_d) vs. 1/T for the interaction of BOFP with SaFtsZ (filled circles), EfsFtsZ (filled triangles),
EfmFtsZ (open triangles), SpyFtsZ (open diamonds), SagFtsZ (filled diamonds), and SpnFtsZ (open squares). The solid lines reflect linear fits of the experimental data points with Eq. 3.3.
Figure 3.4  FtsZ visualization in the Gram-positive bacterial pathogens *S. aureus* NRS705 (a,b),...
E. faecalis ATCC 29212 (c,d), E. faecium ATCC 19434 (e,f), S. pyogenes ATCC 19615 (g,h), S. agalactiae ATCC 12386 (i,j), and S. pneumoniae ATCC 49619 (k,l). Differential interference contrast (DIC) and fluorescence micrographs of the indicated bacterial cells treated for 5 minutes with 1 µg/mL BOFP just prior to visualization. The arrows in panels (b), (d), (f), (h), (j), and (l) highlight representative FtsZ Z-rings at midcell labeled by BOFP.
Figure 3.5  Fluorescence anisotropy profiles of 0.1 µM BOFP as a function of increasing concentrations of EcFtsZ (a), KpFtsZ (b), PaFtsZ (c), or AbFtsZ (d). Acquisition and display parameters are as described in the legend to Figure 3.2b. Panel (e) shows plots of ln(1/ K_d) vs. 1/T.
for the interaction of **BOFP** with EcFtsZ (open circles), KpFtsZ (open squares), PaFtsZ (filled diamonds), and AbFtsZ (filled circles). The solid lines reflect linear fits of the experimental data points with Eq. 3.3.
Figure 3.6 FtsZ visualization in the Gram-negative bacterial pathogens *E. coli* ATCC 25922 (a,b), *K. pneumoniae* ATCC 13883 (c,d), *P. aeruginosa* ATCC 27853 (e,f), and *A. baumannii* ATCC 19606 (g,h). Differential interference contrast (DIC) and fluorescence micrographs of the indicated bacterial cells treated for 5 minutes with 1 µg/mL BOFP in the presence of pentamidine isethionate (at 0.875 mg/mL for *E. coli* and 3.5 mg/mL for the other three strains) just prior to visualization. The arrows in panels (b), (d), (f), and (h) highlight representative FtsZ Z-rings at midcell labeled by BOFP.
Figure 3.7 Visualization of the impact of treatment with 1 on FtsZ localization in *S. aureus*
NRS705, *E. coli* N43, and *K. pneumoniae* ATCC 10031. Differential interference contrast (DIC) and fluorescence micrographs of the indicated bacterial cells treated for 3 hours with either DMSO vehicle (a-f) or 1 (g-l) at 4× MIC (1 µg/mL for *S. aureus* and 4 µg/mL for *E. coli* and *K. pneumoniae*). Just prior to visualization, cells were labeled for 5 minutes with 1 µg/mL BOFP in the absence (for *S. aureus*) or presence of pentamidine isethionate (at 0.875 mg/mL for *E. coli* and 3.5 mg/mL for *K. pneumoniae*). The arrows in panels (b), (d), and (f) highlight representative FtsZ Z-rings at midcell labeled by BOFP.
Figure 3.8 Comparison of BOFP and induced expression of the FtsZ-mCherry fusion protein for visualization of the impact of treatment with 1 on FtsZ localization in the MRSA LAC FtsZ-mCherry strain. Differential interference contrast (DIC) and fluorescence micrographs of the bacterial cells treated for 3 hours with either DMSO vehicle (a-d) or 1 (e-h) at 4× MIC (0.25 µg/mL). Just prior to visualization, cells were labeled for 5 minutes with 1 µg/mL BOFP. The arrows in panel panels (b), (c), and (d) highlight representative FtsZ Z-rings at midcell as visualized either by induced expression of FtsZ-mCherry or by labeling with BOFP.
### Table 3.1 Equilibrium affinity constants and thermodynamic parameters for the binding of **BOFP** to FtsZ proteins from Gram-positive and Gram-negative bacteria.

<table>
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<tbody>
<tr>
<td><strong>Gram-Positive:</strong></td>
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<tr>
<td><em>S. aureus</em> (SaFtsZ)</td>
<td>0.88 ± 0.08</td>
<td>1.66 ± 0.12</td>
<td>3.14 ± 0.13</td>
<td>-10.2 ± 0.3</td>
<td>-7.8 ± 1.0</td>
<td>-7.8 ± 0.1</td>
</tr>
<tr>
<td><em>E. faecalis</em> (EfsFtsZ)</td>
<td>1.72 ± 0.06</td>
<td>3.05 ± 0.25</td>
<td>4.62 ± 0.12</td>
<td>-7.9 ± 1.0</td>
<td>-1.2 ± 3.3</td>
<td>-7.6 ± 0.1</td>
</tr>
<tr>
<td><em>E. faecium</em> (EfMftsZ)</td>
<td>2.50 ± 0.14</td>
<td>2.62 ± 0.08</td>
<td>3.14 ± 0.22</td>
<td>-1.9 ± 0.6</td>
<td>+19.2 ± 1.9</td>
<td>-7.8 ± 0.1</td>
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<tr>
<td><em>S. pyogenes</em> (SpyftsZ)</td>
<td>0.91 ± 0.06</td>
<td>1.31 ± 0.08</td>
<td>1.55 ± 0.08</td>
<td>-4.3 ± 1.1</td>
<td>+12.8 ± 3.7</td>
<td>-8.2 ± 0.1</td>
</tr>
<tr>
<td><em>S. agalactiae</em> (SagftsZ)</td>
<td>0.62 ± 0.05</td>
<td>1.02 ± 0.09</td>
<td>1.31 ± 0.04</td>
<td>-6.6 ± 0.6</td>
<td>+5.4 ± 2.0</td>
<td>-8.2 ± 0.1</td>
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<tr>
<td><em>S. pneumoniae</em> (SpnftsZ)</td>
<td>3.02 ± 0.30</td>
<td>3.49 ± 0.25</td>
<td>3.81 ± 0.69</td>
<td>-1.9 ± 0.3</td>
<td>+18.7 ± 1.2</td>
<td>-7.7 ± 0.1</td>
</tr>
<tr>
<td><strong>Gram-Negative:</strong></td>
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<tr>
<td><em>E. coli</em> (EcFtsZ)</td>
<td>0.22 ± 0.03</td>
<td>0.28 ± 0.02</td>
<td>0.44 ± 0.04</td>
<td>-5.6 ± 0.8</td>
<td>+11.2 ± 2.7</td>
<td>-9.0 ± 0.1</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (KpFtsZ)</td>
<td>0.42 ± 0.05</td>
<td>0.58 ± 0.04</td>
<td>0.82 ± 0.04</td>
<td>-5.4 ± 0.1</td>
<td>+10.5 ± 0.1</td>
<td>-8.6 ± 0.1</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (PaFtsZ)</td>
<td>0.23 ± 0.02</td>
<td>0.36 ± 0.06</td>
<td>0.58 ± 0.06</td>
<td>-7.7 ± 0.2</td>
<td>+3.8 ± 0.6</td>
<td>-8.8 ± 0.1</td>
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<tr>
<td><em>A. baumannii</em> (AbFtsZ)</td>
<td>0.40 ± 0.03</td>
<td>0.55 ± 0.02</td>
<td>0.68 ± 0.04</td>
<td>-4.2 ± 0.6</td>
<td>+14.5 ± 2.1</td>
<td>-8.8 ± 0.1</td>
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$^aK_d$ values (determined at 15, 25, and 37 °C) were derived from non-linear least squares fits of the fluorescence anisotropy profiles shown in Figures 3.2, 3.3, and 3.5 with Eq. 3.1, with the indicated uncertainties reflecting the standard deviation of the fitted curves from the experimental data points. $^b\Delta H$ and $^b\Delta S$ values were derived from linear fits of the ln($1/K_d$) vs. 1/T plots shown in Figures 3.3 and 3.5 with Eq. 3.3, with the indicated uncertainties reflecting the standard deviation of the fitted lines from the experimental data points. $^c\Delta G$ values were calculated at T = 310 K (37 °C) using Eq. 3.2 and the corresponding values of $K_d$, with the indicated uncertainties reflecting the maximal errors as propagated through that equation.
Table 3.2 Data collection and refinement statistics for the crystal structures of SaFtsZ$_{12-316}$ in complex with the R enantiomers of 2 and BOFP.

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<th>Data Set</th>
<th>SaFtsZ Complex with 2</th>
<th>SaFtsZ Complex with BOFP</th>
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<tr>
<td>PDB entry</td>
<td>6KVP</td>
<td>6KVQ</td>
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**Data Collection**
- X-ray source: SPring-8 BL44XU
- wavelength: 0.900
- space group: C2

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<th>Unit -Cell Parameters</th>
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<th>6KVQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$, $b$, $c$ (Å)</td>
<td>70.49, 51.74, 86.74</td>
<td>72.27, 49.69, 88.59</td>
</tr>
<tr>
<td>$\beta$ (deg)</td>
<td>108.65</td>
<td>111.24</td>
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<tr>
<td>resolution (Å)</td>
<td>50.0 - 1.40 (1.42 - 1.40)</td>
<td>36.1 - 1.60 (1.66 - 1.60)</td>
</tr>
<tr>
<td>total reflections</td>
<td>360,570</td>
<td>265,092</td>
</tr>
<tr>
<td>unique reflections</td>
<td>58,167</td>
<td>38,791</td>
</tr>
<tr>
<td>completeness (%)</td>
<td>99.2 (100.0)</td>
<td>99.8 (100.0)</td>
</tr>
<tr>
<td>$I/\sigma$</td>
<td>26.7 (2.4)</td>
<td>15.9 (2.0)</td>
</tr>
<tr>
<td>$R_{merge}$ (%)</td>
<td>7.2 (70.4)</td>
<td>6.3 (82.2)</td>
</tr>
<tr>
<td>CC$_{1/2}$ (%)</td>
<td>(87.1)</td>
<td>(71.9)</td>
</tr>
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</table>

**Refinement**
- resolution (Å): 41.1 - 1.40 | 36.1 - 1.60
- $R_{work}/R_{free}$ (%): 14.5/17.7 | 19.0/21.4
- no. of chain in the asymmetric unit: 1 | 1

**No. of Atoms**
- protein: 2,387 | 2,234
- ligand: 59 | 80
- water: 340 | 165

**Average B-Factors (Å$^2$)**
- protein: 20.6 | 31.7
- ligand: 14.6 | 28.2
- water: 34.8 | 37.8

**rmsd from Ideal**
- bond length (Å): 0.007 | 0.008
- bond angle (deg): 1.43 | 1.28

**Ramachandran Plot (%)**
- favored: 97.9 | 98.4
- allowed: 2.1 | 1.6
- outlier: 0 | 0

*Values in parentheses are for the highest resolution shells.*
FUTURE DIRECTIONS

Given the highly conserved nature of the FtsZ protein among bacterial species, we demonstrate that FtsZ inhibitors can interact with purified FtsZ from both Gram-positive and Gram-negative bacterial pathogens. In addition, the studies presented here demonstrate the utility of FtsZ inhibitors against antibiotic-resistant bacterial pathogens.

While FtsZ inhibitors are capable of blocking cell division Gram-negative bacterial pathogens, they require the assistance of a membrane permeabilizer, such as pentamidine, in order to penetrate the outer membrane barrier and reach the cytoplasmic space where FtsZ is located (as described in Chapter 3). This barrier presents a challenge not only for FtsZ inhibitors but for many potential antibiotics as well.

The outer membrane of the Gram-negative bacterium acts as a natural barrier, preventing many antibiotics from reaching their intracellular targets (118). In addition to this barrier, another major source of antibiotic-resistance in Gram-negative bacterial pathogens is efflux pumps. These pumps are known to cause resistance to many antibiotics by preventing the drugs from reaching their intracellular targets (119-121). One potential strategy for overcoming these barriers that has demonstrated promising results is the conjugation of the antibiotic to a siderophore (122). This approach enables the drug to “hijack” the iron chelating and internalization pathway of the bacterial cell and thus gain access into the intracellular space. As described in chapter 3, we have identified a region on a FtsZ inhibitor to which other chemical groups can be tethered without compromising FtsZ targeting activity. This finding open the door to conjugation of the FtsZ inhibitor with complex molecules like siderophores. Similarly, other important chemical entities that can facilitate the entry of FtsZ inhibitors into the cell could be conjugated to the inhibitor.
Such entities could include cationic membrane permeabilizers like pentamidine or efflux-pump inhibitors.

As shown in Chapter 1, FtsZ inhibitors are appealing new agents that can potentially resurrect antibiotics that have lost efficacy due to resistance. By developing new combinatorial treatments, we can extend the shelf-life of not only the FtsZ inhibitors themselves but also existing antibiotics for years to come.
APPENDIX A

Supplementary Material for Chapter I

SUPPLEMENTAL FIGURES

Figure 1.S1 Chemical structure of TXA707.
**Figure 1.S2** (A-C) Fluorescence anisotropy of 1 µM Bocillin in the presence of the indicated β-lactam antibiotic and 2 µM of either PBP1 (A), PBP2 (B), or PBP3 (C). (D) Initial anisotropy velocity of 1 µM Bocillin in the presence of the indicated β-lactam and 50 µM PBP4. The solid lines reflect the nonlinear least squares fits of the data with Eq. 1.1. Experimental conditions were described in the legends to Figures 1.1 (for PBP1, PBP2, and PBP3) and 1.2 (for PBP4).
Figure 1.S3  Time-kill curves for MRSA COL showing synergy between TXA707 and cefradine (A), ticarcillin (B), ertapenem (C), or cefotaxime (D). Bacteria were treated with DMSO vehicle (black), β-lactam alone at 0.008× MIC (violet), TXA707 alone at 0.5× MIC (red), or a combination of β-lactam at 0.008× MIC and TXA707 at 0.5× MIC (blue).
### Table 1.S1 Oligonucleotide primers used for cloning of *S. aureus* PBPs in *E. coli*

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<th>Name</th>
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<tr>
<td>pbp1-HiFi-F</td>
<td>TCAGTGGTGTTGGTGGTGGTGGGACTTATCCTTGGTC</td>
</tr>
<tr>
<td>pbp1-HiFi-R</td>
<td>TTTAAGAAGGAGATATACATGTGATTACTGGACATTCTAA</td>
</tr>
<tr>
<td>pbp2-HiFi-F</td>
<td>TTTAAGAAGGAGATATACATATGGGTTGGAAGCACCCTGTT</td>
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<tr>
<td>pbp2-HiFi-R</td>
<td>TCAGTGGTGTTGGTGGTGGTGGTGAATACCTGTTAATCACCC</td>
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<tr>
<td>pbp3-HiFi-F</td>
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</tr>
<tr>
<td>pbp3-HiFi-R</td>
<td>CAGTGGTGTTGGTGGTGGTGGCAGGTTTGTCTTTTTATCAATTTTAC</td>
</tr>
<tr>
<td>pbp4-HiFi-F</td>
<td>CTTAAGAAGGAGATATACATATGCTGCAAGCTACTA</td>
</tr>
<tr>
<td>pbp4-HiFi-R</td>
<td>CAGTGGTGTTGGTGGTGGTGGTGCTGAGGTTCATTTTCCCACATACCTTTTA</td>
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The underline sequence reflects the vector complementary sequence
**Figure 2.S1**  Growth curves associated with wild-type and genetically modified MRSA LAC strains.
Figure S2.2  Impact of TXA707 treatment on the cell diameter of MRSA LAC cells expressing a FtsZ-mCherry fusion protein (LAC-Fch) or both FtsZ-mCherry and an sfGFP fusion protein of PBP1, PBP2, PBP3, or PBP4 (LAC-FchP1GFP, LAC-FchP2GFP, LAC-FchP3GFP, and LAC-FchP4GFP, 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-Fch, LAC-FchP1GFP, LAC-FchP2GFP, LAC-FchP3GFP, and LAC-FchP4GFP, respectively) and TXA707-treated cells (n = 315, 531, 368, 326, and 326 for LAC-Fch, LAC-FchP1GFP, LAC-FchP2GFP, LAC-FchP3GFP, and LAC-FchP4GFP, 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 2.S3** Western blot analysis of the mouse anti-MRSA monoclonal antibody (RayBiotech).

Lane 2 contains purified *S. aureus* PBP2a (SaPBP2a) at 0.5 µg/mL, 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 500 µg/mL total protein.
Figure 2.S4 DIC and fluorescence micrographs of MRSA LAC-F_CbP2_GFP 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 fluorescence 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. 2.S2.
**SUPPLEMENTAL TABLES**

**Table 2.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

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<th>Ceftriaxone</th>
<th>Cefotaxime</th>
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<th>Vancomycin</th>
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<td>1</td>
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Table 2.S2  Plasmids used in this study

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<td>J. L. Bose&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pJB38-sfGFP-PBP1</td>
<td>Construction of <em>sfgfp-pbpA</em> allele</td>
<td>This study</td>
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<tr>
<td>pJB38-sfGFP-PBP2</td>
<td>Construction of <em>sfgfp-pbpB</em> allele</td>
<td>This study</td>
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<tr>
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<td>Construction of <em>sfgfp-pbpC</em> allele</td>
<td>This study</td>
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<td>pJB38-PBP4-sfGFP</td>
<td>Construction of <em>pbpD-sfgfp</em> allele</td>
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<td>pJB38-mCherry-PBP2a</td>
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<td>This study</td>
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<tr>
<td>pJB38-PBP2a-mCherry</td>
<td>Construction of <em>mecA-mCherry</em> allele</td>
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<tr>
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<td>pmCherry</td>
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<sup>a</sup>Reference (90).

<sup>b</sup>Reference (123).
### Table 2.S3  DNA fragments (P1, P2, and P3) used in the preparation of the allelic exchange vectors for each listed strain

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<td>Primers&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pjbB up-R</td>
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<td>pjbC up-R</td>
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<sup>a</sup>The sequence of each oligonucleotide primer is listed in Table S4.
**Table 2.S4** Oligonucleotide primers used in this study

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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 *mecA* 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 *mecA* 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 (69), 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).
Figure 3.S1  Fluorescence anisotropy of **BODIPY FL-COOH** (0.1 µM) or **BOFP** (0.1 µM) alone or in the presence of SaFtsZ (10 µM), SpyFtsZ (10 µM), KpFtsZ (2 µM), or PaFtsZ (2 µM). Anisotropy measurements were conducted at 37 °C in solution containing 50 mM Tris-HCl (pH 7.6) and 50 mM KCl.
Figure 3.S2  Fluorescence anisotropy of BOFP (0.1 µM) alone or in the presence of SaFtsZ (10 µM), with the latter also being shown in the presence of GMPCPP (0.1 mM), MgCl$_2$ (10 mM), or both. Anisotropy measurements were conducted at 37 °C in solution containing 50 mM Tris-HCl (pH 7.6) and 50 mM KCl.
Figure 3.S3  SaFtsZ forms a polymer in the crystal. In this trimeric representation, the R enantiomer of BOFP (green) and GDP (cyan) are depicted as stick models and the protein molecules (gray) are depicted as cartoon models.
Figure 3.S4  Comparison of BODIPY FL-COOH and BOFP for visualization of FtsZ in S. aureus NRS705 (MRSA).  Differential interference contrast (DIC) and fluorescence micrographs of the bacteria treated for 5 minutes with 1 µg/mL BODIPY FL-COOH (a,b) or 1 µg/mL BOFP (c,d) just prior to visualization.  The arrows in panel (d) highlight representative FtsZ Z-rings at midcell labeled by BOFP.
Figure 3.S5 Impact of pentamidine isethionate on FtsZ visualization with BOFP in the Gram-negative bacterial pathogen *K. pneumoniae* ATCC 13883. Differential interference contrast (DIC) and fluorescence micrographs of the bacteria treated for 5 minutes with 1 µg/mL BOFP in the absence (a,b) or presence (c,d) of pentamidine isethionate (at 3.5 mg/mL) just prior to visualization. The arrow in panel (d) highlights a representative FtsZ Z-ring at midcell labeled by BOFP.
Figure 3.S6  Comparison of BODIPY FL-COOH and BOFP for visualization of FtsZ in *K. pneumoniae* ATCC 13883. Differential interference contrast (DIC) and fluorescence micrographs of the bacteria treated for 5 minutes with 1 µg/mL BODIPY FL-COOH (a,b) or 1 µg/mL BOFP (c,d) in the presence of pentamidine isethionate (at 3.5 mg/mL) just prior to visualization. The arrows in panel (d) highlight representative FtsZ Z-rings at midcell labeled by BOFP.
Figure 3.S7  Visualization of the impact of treatment with **PC190723** or **TXA707** on FtsZ localization in *S. aureus* NRS705 (MRSA). Differential interference contrast (DIC) and fluorescence micrographs of the bacterial cells treated for 3 hours with DMSO vehicle (a,b), **PC190723** (c,d) at 4× MIC (2 µg/mL), or **TXA707** (e,f) at 4× MIC (4 µg/mL). Just prior to visualization, cells were labeled for 5 minutes with 1 µg/mL **BOFP**. The arrows in panel (b) highlight representative FtsZ Z-rings at midcell labeled by **BOFP**.
### Table 3.S1  
Sequences of the oligonucleotide primers used for cloning the FtsZ genes into the pET-22b(+) expression vector.

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*The underlined sequence reflects the vector complementary sequence, while the bold sequence encodes the 6x His-tag.*
Table 3.S2  Sequences of the oligonucleotide primers used for the generation of the MRSA LAC strain expressing the FtsZ-mCherry fusion protein.

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<td>pCM11-R</td>
<td>AATATCAGAAGCTTGGCCTTGA</td>
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<tr>
<td>pMUTIN-FtsZ-F</td>
<td>GGAGGTGATCTAGAGTCAGGCAAATAAATGTTAGGAGGAATTTA</td>
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<tr>
<td>pMUTIN-FtsZ-R</td>
<td>TGCTCACCATGAGGCGCGCGAGGAACGTTCTTTGCTTTCTTGGAA</td>
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<tr>
<td>mCherry-F</td>
<td>CGCGCGCTCCATGGAAGCAGGGCGGA</td>
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<tr>
<td>mCherry-R-pMUTIN</td>
<td>ATTAGGCAGGGCTCGACTAGACTACTTTGCTACGCTCGTCCATG</td>
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<tr>
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<td>TCTAGTCAGCCGCCCTAATGAGCGGCTTTTTTC</td>
</tr>
<tr>
<td>pMUTIN-R</td>
<td>CTGACTCTAGATCGACCTCCCTTAAGCTTTA</td>
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<tr>
<td>pLL39-FtsZ-F</td>
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<tr>
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<tr>
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<td>TGCAGGCATGCAAGCTTTTTATTACCTAC</td>
</tr>
<tr>
<td>pLL39-R</td>
<td>AATTCGATTAGATCTAGCTCTCCCCGGA</td>
</tr>
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</table>

*The underlined sequence reflects the 5 amino acid linker used to fuse FtsZ to mCherry.*
SUPPLEMENTAL METHODS

General protocol for the cloning, expression, and purification of the FtsZ proteins from S. aureus, E. faecium, S. pyogenes, S. agalactiae, K. pneumoniae, P. aeruginosa, and A. baumannii. The genomic DNA for each organism was extracted from overnight cultures using the DNeasy UltraClean Microbial Kit (QIAGEN). The ftsZ gene for each organism was amplified from the genomic DNA using Q5 High-Fidelity DNA polymerase (New England Biolabs) and the appropriate primers (the sequences of which are listed in Table S1) designed to introduce a 6x His-tag at the C-terminus of each recombinant FtsZ protein. The expression vector pET-22b(+) (Novagen-EMD Chemicals) was linearized using the restriction enzymes NdeI and HindIII. The linearized plasmid and the amplified PCR products were combined and assembled using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs), with the resulting recombinant plasmid being used to transform E. coli NEB5-α. The transformed E. coli NEB5-α 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 plasmids were then isolated from E. coli NEB5-α and after verification of their sequences, were subsequently transformed into E. coli BLR (DE3) cells.

The transformed E. coli BLR (DE3) were grown on LB agar plates containing 100 µg/mL ampicillin. Single colonies were isolated and grown at 37 °C overnight in 50 mL of LB broth supplemented with 100 µg/mL ampicillin. The overnight cultures were diluted 1:100 into 2-4 L of LB broth containing 100 µg/mL ampicillin and incubated at 37 °C until the OD_{600} reached 0.6. The cultures expressing the FtsZ proteins from S. agalactiae and E. faecium were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37 °C for an additional 5 hours. The cultures expressing all other FtsZ proteins were induced with 1 mM IPTG and
incubated at 15 °C for 18 hours. The cells were harvested by centrifugation at 5,000 g for 15 minutes at 4 °C and the cell pellets were then resuspended in 10 mL of buffer A {50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 20 mM imidazole} supplemented with 10% (v/v) glycerol and stored at -80 °C.

The cells were lysed by ultra-sonication (with a Qsonica Q500 sonicator equipped with a ½-inch probe) for 15 minutes at 0 °C, with an on/off cycle of 10 seconds at 60 W. The lysates were centrifuged at 10,000 g for 30 minutes at 4 °C and the resulting supernatants were added to 7 mL of TALON metal affinity resin (Clonetech Laboratories) and then shaken for 1 hour at 4 °C. The resin was then washed with 50 mL of buffer A and loaded into a gravity flow column. The protein was then eluted with 15 mL of elution buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, and 250 mM imidazole. Protein fractions were collected in volumes of 500 µL each. Each fraction was analyzed by SDS-PAGE and those containing the FtsZ protein of interest were combined. The combined fractions were dialyzed overnight at 4 °C in 4 L of buffer containing 50 mM Tris-HCl (pH 7.6) and 50 mM KCl, with the resulting dialysates being concentrated to a volume of 0.2-1 mL using Amicon Ultra-4 10K filters (EMD Millipore). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher). The protein solutions were then aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C.

**Generation of a MRSA LAC strain expressing an inducible FtsZ-mCherry fusion protein.**

We constructed a MRSA strain in which a FtsZ-mCherry fusion protein is ectopically expressed under control of the isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible Pspac promoter. A list of primers required for the amplification of each DNA fragment is listed on Table 3.S2. In order to control the expression of FtsZ-mCherry, we introduced a multicopy plasmid constitutively expressing the LacI repressor. For generation of this initial plasmid, we first amplified the lacI
repressor gene from the pMUTIN-HA(124) plasmid using Q5 High-Fidelity DNA polymerase and the pCM11-LacI-F and pCM11-LacI-R primers. The multicopy vector pCM11(125) was then amplified using Q5 High-Fidelity DNA polymerase and the pCM11-F and pCM11-R primers. This process resulted in the deletion of the lacO sequence. The amplicons resulting from the two amplification reactions described above were then combined and assembled using the NEBuilder HiFi DNA Assembly Cloning Kit to form the plasmid pCM11-lacI. pCM11-lacI was then transformed into E. coli NEB5-α and its sequence subsequently verified. pCM11-lacI was then electroporated into S. aureus RN4220 (an MSSA strain) and subsequently transduced into S. aureus LAC (a USA300 MRSA strain) using the bacteriophage 80α(88), thereby resulting in the strain MRSA LAC lacI.

We then proceeded to generate the fusion protein FtsZ-mCherry by amplifying the FtsZ gene from the MRSA LAC genome using the pMUTIN-FtsZ-F and pMUTIN-FtsZ-R primers. The mCherry reporter gene was amplified from the plasmid pmCherry (Clonetech Laboratories) using the mCherry-F and mCherry-R-pMUTIN primers. The pMUTIN-HA plasmid was then amplified using the pMUTIN-F and pMUTIN-R primers, which resulted in the deletion of 10 nucleotides in the multiple cloning site. The amplicons resulting from the three amplification reactions described above were then combined and assembled using the NEBuilder HiFi DNA Assembly Cloning Kit to form the plasmid pMUTIN-FtsZ-mCherry, which now contains the gene encoding the FtsZ-mCherry fusion protein under control of the Pspac promoter. pMUTIN-FtsZ-mCherry was then transformed into E. coli NEB5-α and its sequence subsequently verified.

To integrate the gene encoding the FtsZ-mCherry fusion protein into the chromosome of MRSA LAC we amplified the DNA sequence containing the Pspac promoter and the fisZ-mCherry gene from the pMUTIN-FtsZ-mCherry plasmid using the pLL39-FtsZ-F and pLL39-FtsZ-R
primers. The single-copy integration vector pLL39(126) was then amplified using the pLL39-F and pLL39-R primers. The amplicons from the two amplification reactions described above were then combined and assembled using the NEBuilder HiFi DNA Assembly Cloning Kit to form the pLL39-FtsZ-mCherry plasmid. pLL39-FtsZ-mCherry was then transformed into *E. coli* NEB5-α and its sequence verified. pLL39-FtsZ-mCherry was then electroporated into an MSSA RN4220 strain containing the pLL2787 plasmid that expresses the φ11 *int* gene(126), which in turn resulted in the integration of the DNA sequence containing the Pspac promoter and the *ftsZ*-mCherry gene at the φ11 attB site of the bacterial chromosome. The resulting genome was subsequently transduced into the MRSA LAC *lacI* strain using the bacteriophage 80α, resulting in generation of the MRSA LAC FtsZ-mCherry strain. The presence of the DNA sequence containing the Pspac promoter and the *ftsZ*-mCherry gene in the genome of MRSA LAC FtsZ-mCherry was confirmed by PCR.

**Synthesis of BOFP.** As schematically depicted in figure 3.2, 2-(5-bromo-4-(4-(trifluoromethyl)phenyl)oxazol-2-yl)-2-(3-carbamoyl-2,4-difluorophenoxy)ethyl 3-(5,5-difluoro-7,9-dimethyl-5H-5,6-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoate (BOFP) was synthesized by adding BODIPY FL-COOH (12 mg, 0.04 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (12 mg, 0.06 mmol), and 4-dimethylaminopyridine (DMAP) (5.0 mg, 0.04 mmol) to 3 (20 mg, 0.04 mmol) in CH₂Cl₂ (3 mL). The reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted with CH₂Cl₂ and washed with brine. The organic layer was dried over sodium sulfate and filtered. The filtrate was then concentrated and purified using ISCO column chromatography on silica gel (50% ethyl acetate/hexanes) to give the product (25 mg, 82% yield) as a deep red solid. ¹H NMR (300 MHz, CDCl₃) δ: 8.06 (d, *J* = 8.41 Hz, 1H), 7.68 (d, *J* = 9.0 Hz, 1H), 7.19 (m, 1H), 6.83 (m, 1H), 6.81 (d,
$J = 3.9 \text{ Hz, } 1H), 6.23 \text{ (d, } J = 3.9 \text{ Hz, } 1H), 6.11 \text{ (s, } 2H), 5.86 \text{ (s, } 2H), 5.42 \text{ (dd, } J = 4.8, 7.8 \text{ Hz, } 1H), 4.81 \text{ (dd, } J = 7.8, 11.4 \text{ Hz, } 1H), 4.67 \text{ (dd, } J = 4.8, 11.7 \text{ Hz, } 1H), 3.27 \text{ (t, } J = 7.8 \text{ Hz, } 1H), 2.81 \text{ (t, } J = 7.5 \text{ Hz, } 1H), 2.53 \text{ (s, } 3H), 2.24 \text{ (s, } 3H). \quad ^{13} \text{C NMR (400 MHz, CDCl}_3 \text{) } \delta: 171.91, 161.42, 160.70, 159.70, 156.29, 144.22, 136.49, 135.31, 133.24, 132.81, 127.97, 126.71, 125.58, 125.53, 123.87, 121.68, 120.62, 119.72, 116.49, 111.82, 111.58, 74.37, 63.47, 33.16, 23.81, 14.92, 11.28.

Electrospray ionization (ESI) high-resolution mass spectrometry (HRMS) of BODIPY dyes typically yields $[\text{M+H-F}]^+$ as the most abundant product ion, due to neutral loss of F(127). We also observed $[\text{M+H-F}]^+$ as the most abundant ion of our reaction product, with the ESI HRMS of $\text{C}_{33}\text{H}_{25}\text{BBF}_7\text{N}_4\text{O}_5$ being as follows: $[\text{M+H-F}]^+$ calculated 761.1006, found 761.1025.
REFERENCES


