Measurement of Accumulation of Antibiotics to *Staphylococcus aureus* in Phagosomes

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Abstract

Staphylococcus aureus (*S. aureus*) has evolved the ability to persist after uptake into host immune cells. This intracellular niche enables *S. aureus* to potentially escape host immune responses and survive the action of antibiotics. The elevated tolerance of *S. aureus* to small molecule antibiotics is likely to be multifactorial. We pose that there may be contributions related to permeation into phagosome, which would require translocation across two mammalian bilayers. To empirically test this, we adapted our recently developed permeability assay to determine the accumulation of FDA-approved antibiotics in phagocytic vacuoles. Bioorthogonal reactive handles were metabolically anchored within the surface of *S. aureus,* and complementary tags were chemically added to antibiotics. Following phagocytosis of labeled *S. aureus* cells, we were able to specifically analyze the accumulation levels of antibiotics within the phagosomes of infected macrophages. Our findings enabled the determination of differences between the permeability of antibiotics to extra- and intracellular *S. aureus*, thus providing a roadmap to dissect the contribution of antibiotic permeability to intracellular pathogens.

Introduction

Staphylococcus aureus (*S. aureus*) is a significant human pathogen that can cause a wide range of infections from mild skin infections to severe disease such as sepsis and pneumonia.[1-3] The increasing incidence of drug-resistant *S. aureus* infections, particularly methicillin-resistant *S. aureus* (MRSA), is a major concern.[4-6] MRSA is resistant to many commonly used antibiotics, making it difficult to treat. Overall, the increasing incidence of drug-resistant *S. aureus* infections highlights the importance of understanding the factors that impact the efficacy of therapeutics against bacterial pathogens.

In addition to genetic alterations that result in drug resistant phenotypes, *S. aureus* has evolved another mode of persistence during infection. Host immune cells, such as macrophages and neutrophils, phagocytose bacteria as an anti-bacterial response.[7-11] These immune cells have a variety of potent mechanisms to kill phagocytosed bacteria, including the release of reactive oxygen and nitrogen species (ROS and RNS), the production of antimicrobial peptides and enzymes, and acidification of the phagosome.[12] In response, *S. aureus* subvert these directed challenges by surviving within host cells, particularly immune cells.[7, 13-19] Bacterial cells that undergo intracellular survival can serve as a potential reservoir that seeds reoccurring infections and can persist for days,[8-9, 20] a feature that has been demonstrated both *in vitro* and *in vivo*.[21-23]

Mechanistically, antibiotic tolerance by intracellular *S. aureus* has been previously ascribed to a phenotypic switch to small colony variants.[11, 24-25] An alternative or

complementary mechanism that can promote the persistence of *S. aureus* within immune cells could also be promoted by the reduced accumulation of antibiotics to the subcellular organelle housing these cells. Antibiotics must traverse two membrane bilayers (immune cell plasma membrane and phagosome bilayer) to reach the target pathogen. These additional barriers could potentially diminish the effective concentration of the therapeutic agent within the phagosome, leading to less effective antibacterial activity. Herein, we developed an assay – that can also be readily adopted to several intracellular pathogens – to directly measure the accumulation of the antibiotic to the surface of *S. aureus* present in the phagosomes of macrophages. We demonstrated that structural editing of antibiotics can dictate the abundance of agents within phagocytic vacuoles. Uniquely, these measurements are reflective of antibiotic accumulation at the target cells and not the entire immune cells.

Results and Discussion

Prior efforts to assess intracellular levels of antibiotics have most commonly been approximated by their antibacterial activity (minimum inhibitor concentration, MIC).[21, 26-27] The main challenges in using MIC determinations as a proxy for the concentration of the antibiotics in the phagosome are that tolerant phenotypes (induced following phagocytosis) can alter MIC values regardless of drug concentration and/or phagosome pH can alter drug efficacy.[28] Critically, all prior studies have analyzed the antibiotic concentration in the entire immune cell rather than isolated to the primary subcellular compartment that houses the internalized *S. aureus*, namely the phagosome. The

concentration of the antibiotic inside the phagosome could potentially differ from that in the general cytosol; thus, the whole cell concentration may not reflect the effective level that reaches the target bacterial cells. Aside from MIC determination, antibiotic concentration measurements have also been made based on the inherent fluorescence of those agents, such as in the case of quinolones[21, 29] or by introducing a radioactive isotope.[30-31] Both of these methods pose clear challenges and limitations.

Appreciating that intracellular residence can lower the efficacy of antibiotics, a group at Genetech developed an antibody-antibiotic conjugate that eliminated intracellular *S. aureus* in an *in vivo* model of infection. More specifically, a rifamycin derivative was conjugated to an antibody directed at the wall-teichoic acids (WTA) of *S. aureus,* whereas rifamycin was released upon cleavage by intracellular proteases.[22] Another clever strategy involved the use of a proline-based cell penetrating peptide that was conjugated to kanamycin with affinity towards intracellular bacteria.[32] Similarly, vancomycin was conjugated to an arginine-based cell penetrating peptide, which led to its potentiation.[33] Antimicrobial peptides have also been designed to directly target intracellular bacterial pathogens.[34-35] Together, these efforts highlight the challenges that can arise in the accessibility of antibiotics to phagosomes. We set out to isolate the impact of intracellular residence to the accessibility of antibiotics by systematically determining the permeability of select antibiotics to the cell wall of intracellular *S. aureus* (**Figure 1**).

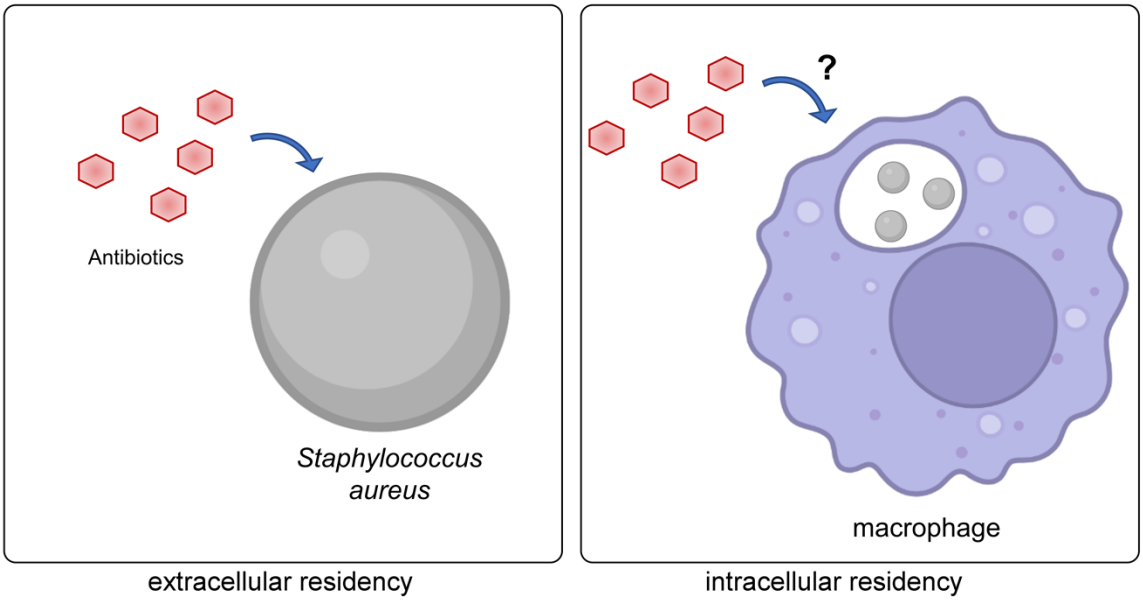

Figure 1. Cartoon representation of antibiotic permeability to extracellular *S. aureus* (left) and intracellular *S. aureus* (right).

The cell wall of *S. aureus* provides a formidable barrier to extracellular threats including antibiotics and anti-bacterial components of the immune system. For Gram-positive bacteria, the cell wall is primarily comprised of a thick layer of peptidoglycan (PG) that displays various surface proteins and extracellular matrices; these biomacromolecules are essential to maintain the overall integrity of the cell.[36] The PG is composed of repeating disaccharide units, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), covalently linked to a short stem peptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala). The stem peptide strands are crosslinked together by transpeptidase (TP) enzymes to create a dense, mesh-like scaffold that surrounds the cell as a single molecule.[37-39] Given that the PG is a critical component to the fitness of the pathogen, many antibiotics target PG biosynthesis enzymes.[40-41]

Our lab[42-52] and others[53-56] have extensively demonstrated the ability to metabolically remodel bacterial PG with synthetic analogs of PG precursors. Further, we demonstrated that such analogs can be incorporated *in vivo* into the cell wall of *S. aureus* infected *C. elegans*[50] and various diverse bacterial species residing in the gut microbiome of a mouse model.[57] More recently, our laboratory described the installation of click handles on the PG to systematically assess how flexibility and size of large biopolymers impact their ability to reach the PG surface.[47, 58] We reasoned that site-specific metabolic installation of a bioorthogonal reactive handle within the PG of *S. aureus* could also be leveraged to analyze the accumulation of antibiotics to *S. aureus* in phagocytic vacuoles in immune cells.

We synthesized a D-amino acid containing dibenzocyclooctyne (DBCO) on the sidechain. Incubation of this amino acid in the growth media of *S. aureus* results in the exchange of the terminal D-alanine on the PG stem peptide with the supplemented unnatural amino acid (**Figure S1**). This installation of DBCO within the PG of *S. aureus* is site selective and does not alter cellular viability. DBCO-tagged bacterial cells are then incubated with macrophages to promote their uptake. Treatment of cells with azide-modified antibiotics (azAbx) post infection is expected to result in the reaction between the DBCO and azAbx *via* strain promoted alkyne-azide cycloaddition (SPAAC).[59-61] A chase step with an azide-modified fluorophore allows reaction with unmodified DBCO epitopes, enabling a fluorescent, live-cell analysis of antibiotic accumulation, whereby cellular fluorescence inversely correlates with antibiotic permeability.

To start, we synthesized a single amino acid with a DBCO conjugated to the sidechain of D-Dap (D-DapD), and metabolic incorporation of D-DapD was measured in bacterial cells. *S. aureus* cells were incubated with culture media containing D-DapD (or its stereocontrol, L-DapD) overnight to allow for incorporation throughout the PG matrix. Bacterial cells were washed to remove unincorporated metabolic tags, followed by incubation with azide-modified rhodamine 110 (R110az), and cellular fluorescence measurement by flow cytometry (**Figure 2A**). Satisfyingly, the fluorescence associated with *S. aureus* cells metabolically labeled with D-DapD were significantly higher than DMSO treated cells (**Figure 2B**). Cells treated with the stereocontrol, L-DapD, exhibited similar fluorescence levels to DMSO treated cells indicating that the DapD was incorporated in the PG scaffold of *S. aureus* in a stereoselective manner. Additionally, incubation with D-DapD did not perturb cell growth (**Figure 2C**). Confocal microscopy showed that the fluorescence pattern was consistent with PG incorporation as the fluorescence remained peripheral and septal (**Figure 2D**).

We further confirmed DBCO installation on the PG of *S. aureus* by isolating the entire PG scaffold (sacculi) from cells that had been treated with D-DapD followed by R110az. Sacculi isolation was performed using standard methods that disrupt the whole cell, leaving the single molecule cell-size sacculus. Isolation of sacculi with the DBCO tag was not performed due to the chemical instability of the triple bond of DBCO in the isolation conditions (acid, high temperatures). Instead, we isolated the stable triazole product formed after the SPAAC reaction. Flow cytometry analysis of the fluorescently tagged

sacculi (SaccuFlow[62]) was performed wherein the sacculi was found to be highly fluorescent (**Figure S2**). Sacculi isolated from DMSO-treated *S. aureus* cells displayed minimal fluorescence levels. As expected, confocal microscopy analysis revealed that the fluorescently labeled sacculi were similar in size and shape compared to whole cell *S. aureus* while also retaining similar labeling patterns (**Figure 2D**). Together, these results established that tagging of the sacculi was successful and the DBCO sites could be specifically labelled by treatment with an azide-modified fluorophore.

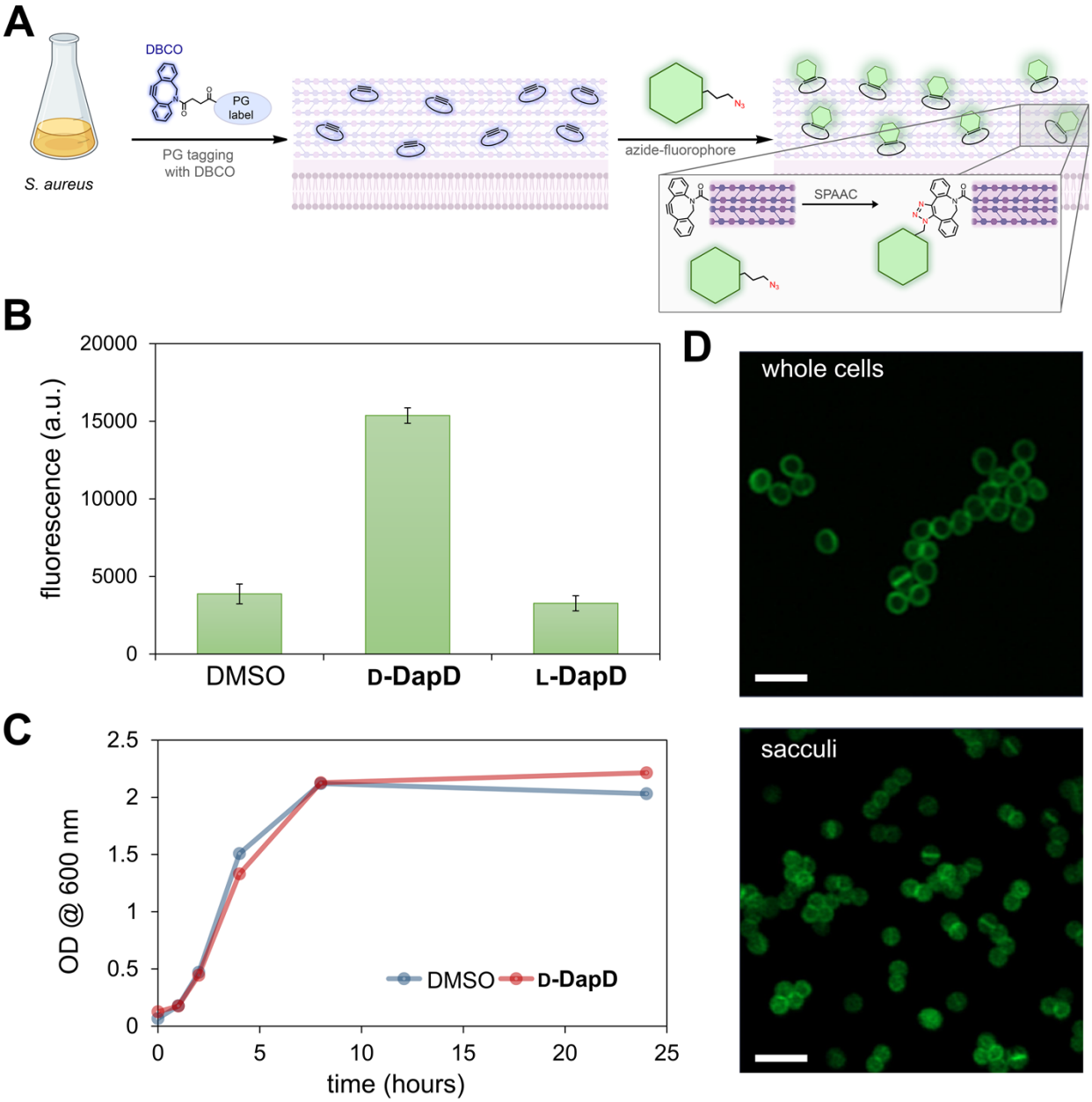

Figure 2. A) Workflow schematic of the PG labeling with a DBCO-displaying agent followed by treatment with an azide-tagged fluorophore. B) Flow cytometry analysis of *S. aureus* treated overnight with 500 μM of D-DapD and incubated for 30 min with 25 μM of R110az. C) Measurement of OD_{600} over time for *S. aureus* cells treated with either DMSO or 500 μM of D-DapD. D) Confocal microscopy analysis of *S. aureus* cells (top)

and isolated sacculi (bottom) after treatment with 500 μM of D-DapD followed by incubation with 25 μM of R110az. Scale bar = 5 μM. Data are represented as mean ± SD (n= 3).

We next set out to test labeling conditions to optimize the dynamic range of the assay. Following the same workflow as described before, *S. aureus* cells were grown overnight in the presence of increasing concentrations of D-DapD, washed, treated with R110az, and analyzed by flow cytometry. As expected, the cellular fluorescence levels of *S. aureus* grown with D-DapD increased in a concentration-dependent manner (**Figure S3A**); this indicates that more D-DapD was covalently linked into the growing PG scaffold as more amino acid was supplemented into the growth media. From these results, the D-DapD concentration of 500 μM was selected for subsequent experiments. We next evaluated the impact of fluorophore concentration on the cellular fluorescence levels. *S. aureus* cells were labeled D-DapD followed by incubation with increasing concentrations of R110az, which resulted in increasing cellular fluorescence levels, as expected (**Figure S3B**). To ensure the timeframe of the assay on the surface of *S. aureus* will enable completion of the SPAAC reaction, a time course of R110az labeling was performed, which indicated that the R110az reacted with the majority of available DBCO sites on the PG layer within 30 minutes (**Figure S3C**). Lastly, to investigate the permeation effects of the fluorophore itself, we examined two additional azide fluorophores: fluorescein azide (Flaz) and coumarin azide (Coaz). Both were demonstrated to be compatible (**Figure S3D**), as indicated by significant fluorescence signals over cells in the absence of the metabolic label.

We then evaluated the ability of the assay to report on the permeability of FDA approved antibiotics onto the PG of *S. aureus*. We synthesized an azAbx panel to make them compatible with our assay workflow (**Figure 3B**). Azides were installed on parts of the antibiotic that were found to minimally perturb their efficacy (**Figure S4**). The azAbx included were rifampicin, erythromycin, linezolid, and ciprofloxacin, which are treatment options for *S. aureus* infections.[63-66] The selected antibiotics have chemical compositions that range in hydrophobicity, polarity, size, and charge to examine how these factors affect their ultimate accumulation into phagosomes. Furthermore, we also tested two variations of ciprofloxacin: one containing the azide modification on the pyridinyl group that has an overall net negative charge and another that has an additional ester modification to return the molecule to a net neutral charge. This modification was done to evaluate the impact of net charge on the permeability of antibiotics to the phagosome. In the pulse step, DBCO-tagged cells were treated with the azAbx for an empirically defined time period. Antibiotics that reach the PG are expected to form a stable and irreversible bond with DBCO. In the chase step, R110az reacts with the remaining intact DBCO sites on the PG. Therefore, antibiotics that readily reach the bacterial pathogens should result in low cellular fluorescence levels (**Figure 3A**).

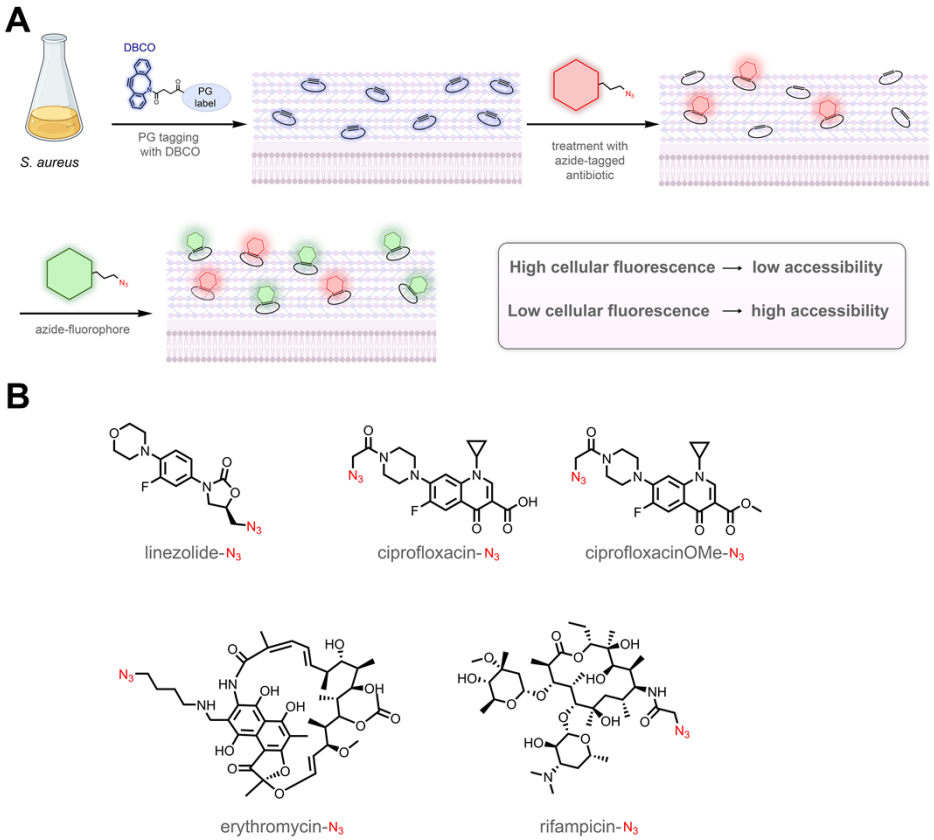

Figure 3. A) Schematic of the general workflow for assessing the permeability of antibiotics to the PG of *S. aureus*. B) Chemical structures of azide modified antibiotics.

To establish the baseline reactivity level of the azAbx without a membrane-permeability barrier, *S. aureus* cells were metabolically tagged with D-DapD and incubated with each individual member of the azAbx library. Following incubation with the azAbx, cells were washed, treated with R110az, and cellular fluorescence was analyzed by flow cytometry. From the data observed, it was found that nonpolar antibiotics, such as linezolid and rifampicin, were able to sieve through the PG and fully react with the DBCO modified PG within 2 h, as indicated by a low fluorescence signal. The small size of linezolid and the

flexible backbone of rifampicin could be, at least in part, the reason for these observations. Conversely, the increased rigidity and polarity of ciprofloxacin and erythromycin could play a role in hindering the permeation of these molecules through the PG; to this end, full reaction was not observed until nearly 8 h (**Figure 4A**). Interestingly, a similar pattern of permeation was observed for the two ciprofloxacin derivatives, indicating that masking the net negative charge of ciprofloxacin-N_3 had negligible effects in terms of reaching DBCO sites within PG of *S. aureus*.

To further ensure whether that the SPAAC reaction between the azAbx and D-DapD was complete within the timeframe of the assay, we modified amine-terminated beads to display DBCO epitopes (**Figure S5A**). As these beads are compatible with flow cytometry, this approach provides a parallel workflow to the live cell analysis but without the potential barrier of the matrix-like PG scaffold. Our results showed that the antibiotics in this panel readily react in the absence of a complex cell wall structure, as indicated by background fluorescence levels observed for all five azAbx within 2 h (**Figure S5B**). These results clearly suggest that the PG itself could pose as a permeation barrier. Sieving through the crosslinked PG has been ascribed to play a role in the activity of antimicrobial peptides[67] and components of the human immune system (e.g., lysozyme and antibodies).[68-69] Early work showed that molecular sieving was operative within isolated sacculi with synthetic polymers[70-71] but systematic studies had not been carried out in live bacterial cells using antibiotics. Sieving within the PG scaffold is likely to be dependent on multiple factors, including the level of crosslinking, which can vary widely.[72]

Given that the estimated crosslinking levels in *S. aureus* can range from 80 to 90%, it is probable that sieving could be particularly prominent.[73-74]

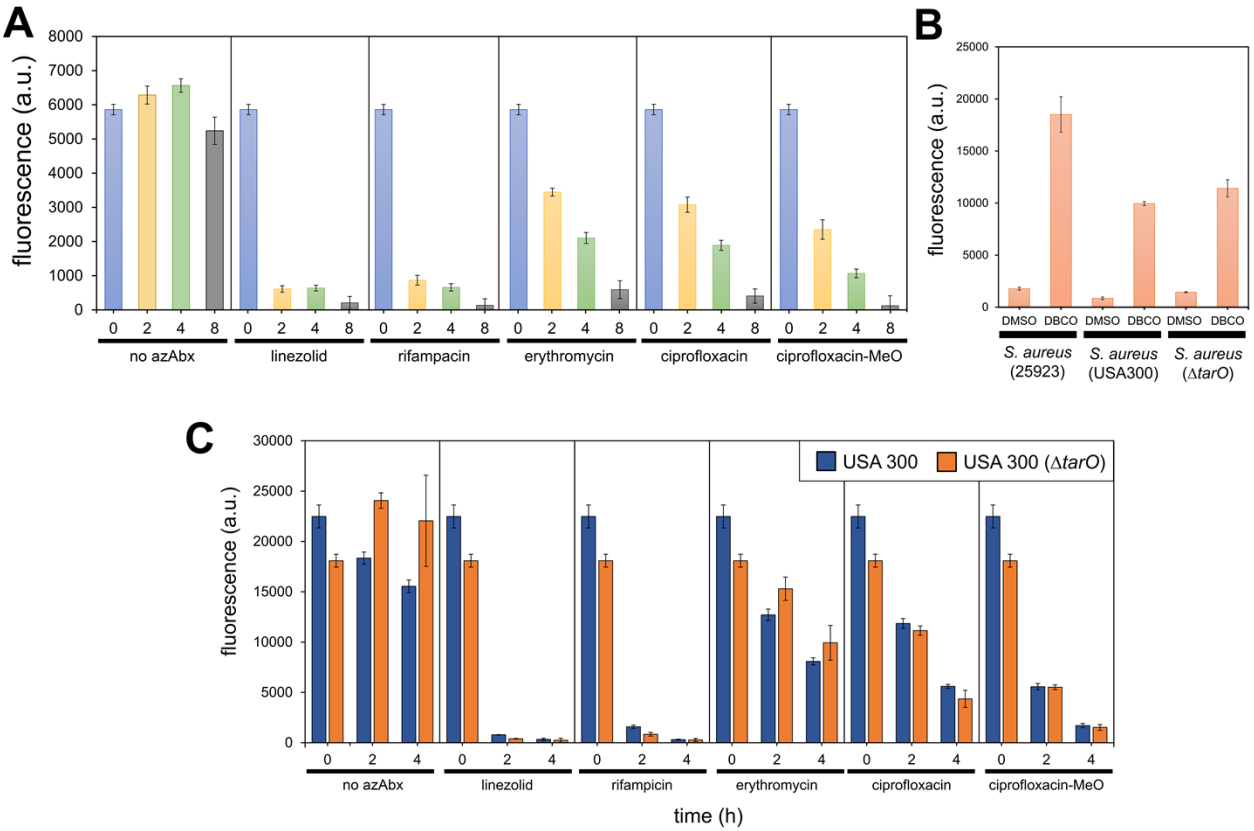

Figure 4. A) *S. aureus* cells were incubated with 500 μM of D-DapD over-night followed by incubation with 25 μM of azABx for indicated time points. Following azAbx incubation, the cells were resuspended in 25 μM of R110az for 30 min and analyzed by flow cytometry. B) *S. aureus* 25923, USA 300, and a WTA deletion strain, dTarO, was incubated overnight with 500 μM D-DapD followed by incubation with 25 μM R110az for 30 min and analyzed by flow cytometry. (C) *S. aureus* cells (USA300 and dTarO) were incubated with 500 μM of D-DapD over-night followed by incubation with 25 μM of azABx for indicated time points. Following azAbx incubation, the cells were resuspended in 25

μM of R110az for 30 min and analyzed by flow cytometry. Data are represented as mean ± SD (n= 3).

To demonstrate the adoptability of the assay across other strains of *S. aureus*, we also performed a similar analysis with MRSA. Satisfyingly, treatment of USA300 *S. aureus* with D-DapD resulted in a high fluorescence signal when incubated with R110az, indicating that D-DapD was incorporated into the PG layer of a methicillin-resistant strain and readily reacted with an azide molecule (**Figure 4B**). Further, we went on to investigate azAbx accessibility to USA300 PG. After incubating D-DapD labeled USA300 with the azAbx, a similar pattern was observed for all azAbx compared to methicillin sensitive *S. aureus* (MSSA), as evidenced by a significant decrease in fluorescence signal compared to DMSO treated cells (**Figure 4C**) after incubation with the azAbx for 8 h. This indicates that the azAbx were able to sieve through the USA300 PG layer.

Further, we aimed to investigate the contribution that PG-anchored, brush-like polysaccharides play on permeability. The surface of *S. aureus* is coated with wall teichoic acids (WTAs), that are anionic glycopolymers known to sterically hinder biomolecules from reaching the surface of the PG.[47, 68, 75] Our group recently described how WTA can be a major determinant of surface accessibility to large polymers (polyprolines and PEG-based), which was determined by also using a combination of PG tags and SPAAC.[47] Analogously, we tested whether WTAs may alter the accessibly of azAbx, which are considerably smaller, to the PG layer. Our data showed no significant difference between the USA300 parental strain and *tarO* deletion strain, which cannot biosynthesize WTA on

the surface of *S. aureus* (**Figure 4B,C**). These results indicate that WTAs do not appear to hinder permeation of smaller molecules to the PG matrix. To the best of our knowledge, this had not been shown before.

With the extracellular benchmarks established, we next sought to test the reportability of molecule accumulation inside phagocytic vacuoles of macrophages. Given the selectivity of the SPAAC reaction, we reasoned that it was possible to isolate accumulation measurements (decoupled from biological activity) inside this cellular compartment. We initially optimized the role of the reporter dye in the absence of azAbx. *S. aureus* were tagged with D-DapD overnight and then incubated with macrophages to promote their uptake. Macrophages containing DBCO-tagged *S. aureus* (or unmodified *S. aureus*) were treated with either 5 μM or 25 μM of R110az (**Figure 5B**). Minimal differences in cellular fluorescence levels were observed at the lower concentration. Upon increasing the dye concentration, a marked difference emerged between DBCO-tagged cells and untagged cells. Additionally, a lower MOI resulted in poorer differences in cellular fluorescence (**Figure S5**). It was determined that MOI of 100 could detect significant changes in R110az uptake for D-DapD *S. aureus* over the unlabeled DMSO control, as indicated by a significant change in fluorescence. Confocal microscopy at two different time points revealed that fluorescence levels were elevated in areas that were consistent with the shape of bacterial cells inside macrophages (**Figure 5C**).

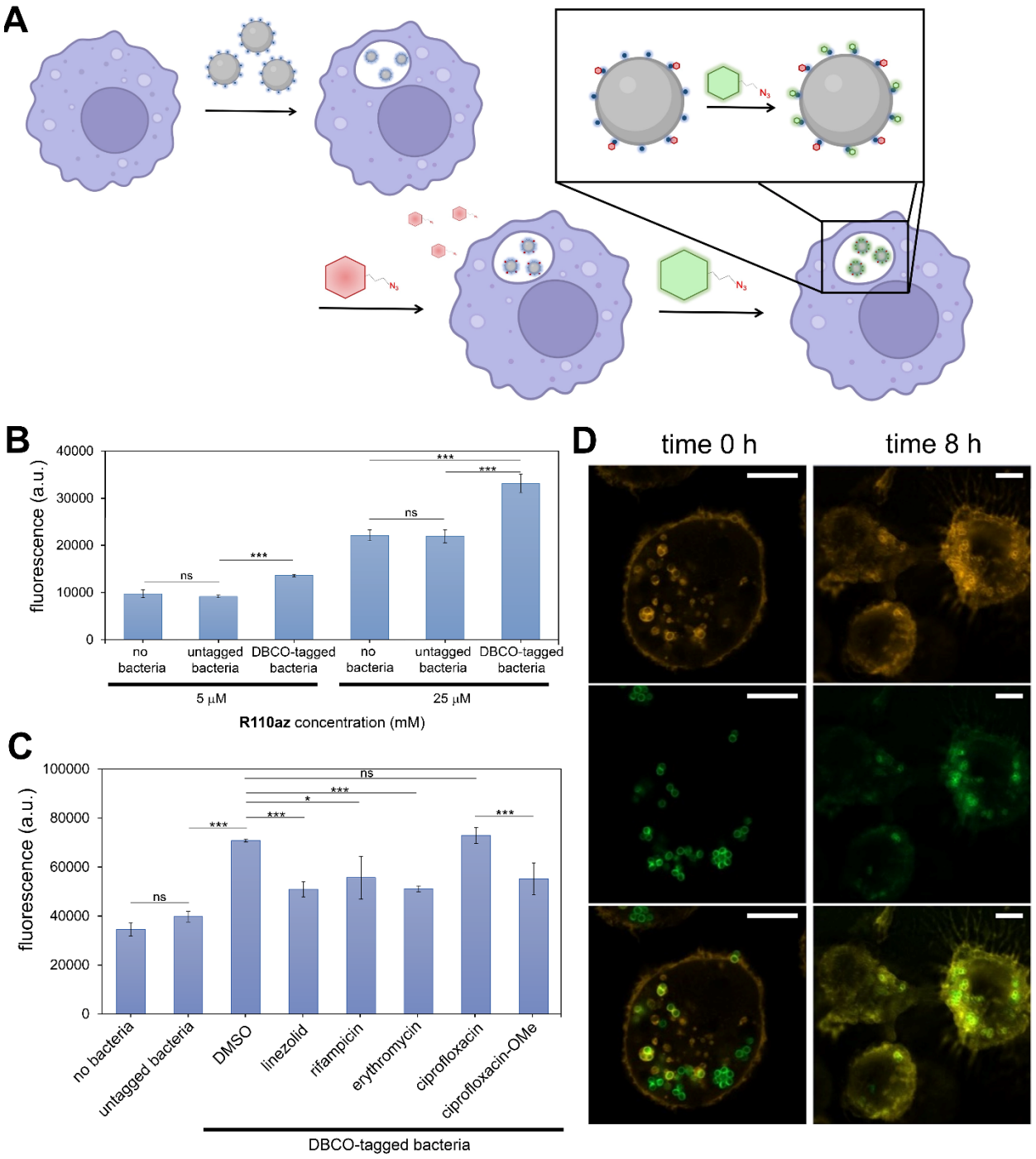

Figure 5. A) Schematic of the assay workflow. *S. aureus* cells that were grown overnight with D-DapD will be infected into macrophages. The azAbx library will be incubated for 8 h with S. aureus infected macrophages, followed by incubation with R110az, and

analyzed by flow cytometry. B) *S. aureus* was grown overnight with 500 μM of D-DapD and infected into J774A macrophages. Following bacterial uptake, varying concentrations of R110az were incubated for 30 min, and cells were analyzed by flow cytometry. C) *S. aureus* cells were grown overnight with 500 μM of D-DapD and infected into J774A at MOI of 100. AzAbx library was incubated with bacteria infected macrophages for 8 h, followed by incubation with 25 μM of R110az and analyzed by flow cytometry. D) Confocal analysis of D-DapD infected J774A macrophages. *S. aureus* was labeled overnight with 500 μM of D-DapD and then immediately reacted with 25 μM of R110az. Fluorescently labeled *S. aureus* was infected into J774A macrophages at MOI 100. Immediately following bacterial uptake, time 0 h samples were fixed and analyzed by confocal microscopy. Time 8 h samples were resuspended in PBS for 8 h, fixed, and analyzed by confocal microscopy. Macrophage membrane was labeled with wheat germ agglutin (WGA) tetramethylrodamine. Scale bar = 10 μM. Data are represented as mean ± SD (n= 3). *P*-values were determined by a two-tailed *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant).

Finally, the accumulation levels of the molecules in the azAbx panel were tested. After macrophage phagocytosis of D-DapD labeled *S. aureus*, cells were incubated with the azAbx panel over an 8 h period (**Figure 5D**). When internalized *S. aureus* labeled with D-DapD was incubated with linezolid, rifampicin, or erythromycin, fluorescence levels were similar that of untagged *S. aureus*; these results are suggestive that these antibiotics can readily reach phagocytic vacuoles. Conversely, ciprofloxacin appeared unable to access

the phagosome, as evidenced by indistinguishable fluorescence levels between ciprofloxacin incubated cells and those in the absence of an azAbx. We reason that the net negative charge of ciprofloxacin-N_3 could play a role in reducing the permeability of this antibiotic across the membrane bilayers. To test this, a derivative was also made in which the carboxylic acid was masked as a methyl ester. Our data showed that a marked improvement in permeability was observed, as the fluorescence for intracellular *S. aureus* treated with ciprofloxacin-MeO was significantly lower than those treated with ciprofloxacin.

The masking of the carboxylic acid of ciprofloxacin (and other fluoroquinolones) as ester has been explored in the past.[76-77] Moreover, our results may indicate that performing phenotypic susceptibility testing *via* MIC against intracellular pathogens may be biased by the inability of an antibiotic to reach the phagosome. We demonstrated that masking groups known to hinder membrane permeability, i.e. ciprofloxacin-MeO, improved antibiotic accumulation to the phagosome, emphasizing the need to develop strategies to improve the permeability profiles of existing antibiotic classes. Therefore, our results highlight the importance of taking antibiotic accessibility to the phagosome into consideration when evaluating drug effectiveness against intracellular pathogens. Furthermore, we successfully demonstrated the applicability of our assay to empirically analyze the permeability of antibiotics to the surface of intracellular *S. aureus.* We anticipate that this platform can serve as foundational precedence to address the permeability of other classes of antibiotics to aid developing effective treatments for persisting intracellular *S. aureus* infections.

Conclusion

We demonstrated that the assay we developed is a suitable approach to assess the permeability of antibiotics to the surface of a Gram-positive pathogen *in vitro* and in the phagocytic vacuoles of macrophages. A select group of FDA approved antibiotics were successfully modified with an azide handle to covalently react with DBCO moieties embedded within the PG of *S. aureus* to give a direct read out of antibiotic accumulation. Understanding the ability of antibiotics to penetrate both the mammalian cell membrane and phagosome is critical in terms of treating intracellular *S. aureus* infections. With our approach, we were able to identify that the overall efficacy of certain classes of antibiotics against intracellular *S. aureus* may be largely impacted by the membrane barrier provided by the phagocytic cell. Therefore, it is critical to consider additional membrane components when it comes to treating intracellular bacterial infections. We anticipate our assay will be valuable in distinguishing the root cause of antibiotic resistance in intracellular pathogens.

Acknowledgement

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Supporting Information

Additional figures, tables, and materials/methods are included in the supporting information file.

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