A Biosynthetic Strategy for Re-engineering the *Staphylococcus aureus* Cell Wall with Non-native Small Molecules

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Staphylococcus aureus is a Gram-positive bacterial pathogen that has become a major public health threat. Most hospital isolates of *S. aureus* are resistant to many if not all available treatments (*1*), and recent reports suggest that more Americans die every year as a result of *S. aureus* infection than due to HIV/AIDS, Parkinson's Disease, or emphysema (*2, 3*). The virulence of this organism is mediated in part by proteins in its cell wall, which enable it to interact with animal cells and tissues and to evade the human immune system (*4, 5*). Flexible strategies for modulating the molecular composition of the *S. aureus* cell wall would therefore be highly desirable and could enable both fundamental and therapeutic applications.

Here we demonstrate for the first time that the cell surface of wild-type *S. aureus* can be re-engineered biosynthetically to incorporate non-native small molecules. Exposure of wild-type bacteria to rationally designed, low molecular weight substrates for the enzyme sortase A (SrtA) (*6*) leads to covalent incorporation of functional small molecules (fluorescein, biotin, or azide) into the *S. aureus* cell wall (Figure 1). Diverse experimental techniques, including epifluorescence and electron microscopy, flow cytometry, mass spectrometry, and biochemical cell wall extraction, are employed to support these conclusions. Furthermore, cell-wall-incorporated azide is exploited as a chemical handle to perform an azide–alkyne cycloaddition reaction on the bacterial cell surface. This report represents both the first example of cell wall engineering of *S. aureus* or any other pathogenic Gram-positive bacteria and has the potential to enable wide-ranging applications.

ABSTRACT *Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterial pathogen that has emerged as a major public health threat. Here we report that the cell wall of *S. aureus* can be covalently re-engineered to contain non-native small molecules. This process makes use of endogenous levels of the bacterial enzyme sortase A (SrtA), which ordinarily functions to incorporate proteins into the bacterial cell wall. Thus, incubation of wild-type bacteria with rationally designed SrtA substrates results in covalent incorporation of functional molecular handles (fluorescein, biotin, and azide) into cell wall peptidoglycan. These conclusions are supported by data obtained through a variety of experimental techniques (epifluorescence and electron microscopy, biochemical extraction, and mass spectrometry), and cell-wall-incorporated azide was exploited as a chemical handle to perform an azide–alkyne cycloaddition reaction on the bacterial cell surface. This report represents the first example of cell wall engineering of *S. aureus* or any other pathogenic Gram-positive bacteria and has the potential for widespread utility.

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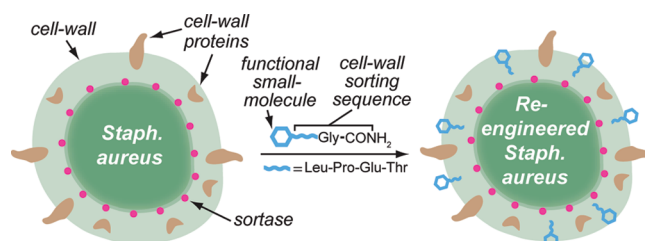david.spiegel@yale.edu.

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Figure 1. Schematic depiction of the reported method for sortase-mediated tagging of the *S. aureus* cell wall. Bacteria are fed peptides containing small molecule tags. Upon penetrating the cell wall, these compounds are recognized by the periplasmic enzyme sortase A (red dots), which cleaves between the C-terminal threonine and glycine residues and covalently attaches the remaining N-terminal portion to lipid II in the forming cell wall, as shown.

RESULTS AND DISCUSSION

Substrate Design and Incorporation into *S. aureus*. Our design of synthetic SrtA substrates was based upon the enzyme's mechanism of action, which involves recognition of a conserved pentapeptide motif, typically LPETG in *S. aureus*, near the C-terminus of various secreted proteins (6). Upon substrate recognition, the enzyme cleaves the threonine—glycine bond, forming an acyl-enzyme intermediate. This complex is attacked by the pentaglycine motif of lipid II, a peptidoglycan precursor, and the resulting adduct is covalently attached to the bacterial cell wall (6). We speculated that since natural SrtA substrates are structurally diverse, the native enzyme present in the bacterial periplasm might be able to integrate LPXTG-tagged small molecules into the cell walls of living bacteria in an analogous manner to protein substrates. Indeed, previous *in vitro* studies have demonstrated that SrtA is capable of catalyzing transpeptidation reactions with non-natural LPXTG-modified substrates (7–11). Also, purified, recombinant SrtA has been shown to catalyze transpeptidation reactions on the surfaces of eukaryotic cells that have been genetically manipulated to express LPXTG-modified surface proteins (12–14). To our knowledge, however, no one has metabolically incorporated small molecule functionality into the *S. aureus* cell wall.

As a convenient means of assessing integration of non-native small molecules into wild-type *S. aureus*, we designed compounds **1**–**6**, which were prepared through Fmoc solid-phase peptide synthesis (Figure 2A). Notably, since the presence of a carboxylic acid at the LPETG C-terminus has been suggested to inhibit SrtA activity (15), we chose to incorporate an amide at this position. Incubating wild-type *S. aureus* cultures with compound **1**, which contains a fluorescein-derivatized lysine residue at the N-terminus of the LPETG pentapeptide, led to highly fluorescent bacteria as measured by epifluorescence microscopy (Figure 2B, panels a and b). Neither control experiments with vehicle alone (Figure 2B, panels c and d), nor with **2**, a fluorescein-containing peptide isomeric to **1** containing a scrambled sorting sequence (Figure 2B, panels e and f), demonstrated detectable cellular fluorescence. This latter control argues against non-specific protein binding as the cause of bacterial fluorescence and is consistent with the proposed mechanism for small molecule integration. Furthermore, an isogenic *S. aureus* strain lacking the SrtA gene (16) exhibited a greatly reduced ability to incorporate **1** into the cell wall, consistent with a critical involvement of SrtA in this process (Figure 2B, panels g and h).

We then quantified the levels of fluorophore incorporation as a function of synthetic small molecule concentration using flow cytometry (Figure 2C). Detectable levels of fluorescein (approximately 1000 molecules cell^{-1}) were observed at concentrations of **1** as low as 125 μM, and the levels of incorporated fluorophores were observed to be roughly proportional to the concentration of **1** in treatment media within a range up to 6500 molecules cell^{-1}. Notably, the native *S. aureus* collagen adhesin protein is surface exposed at a level of approximately 5000 molecules cell^{-1} in wild-type organisms (17). Thus, the amount of incorporated small molecule observed in our studies is comparable to expression levels of this native protein. Importantly, treatment of bacteria with compound **1** neither affects cell viability as measured by replating colonies after peptide treatment at concentrations up to 1 mM (Supplementary Figure 1), nor abrogates endogenous levels of protein A, a cell surface protein and SrtA substrate (Supplementary Figure 2). These results indicate the potential of this technology for the non-disruptive labeling of living bacteria.

Subcellular Localization of SrtA Substrates. To evaluate further the subcellular localization of incorporated small molecules, cell walls were extracted from wild-type *S. aureus* that had been pretreated with either **1** or **2** (Figure 3A). Cell wall extracts from bacteria
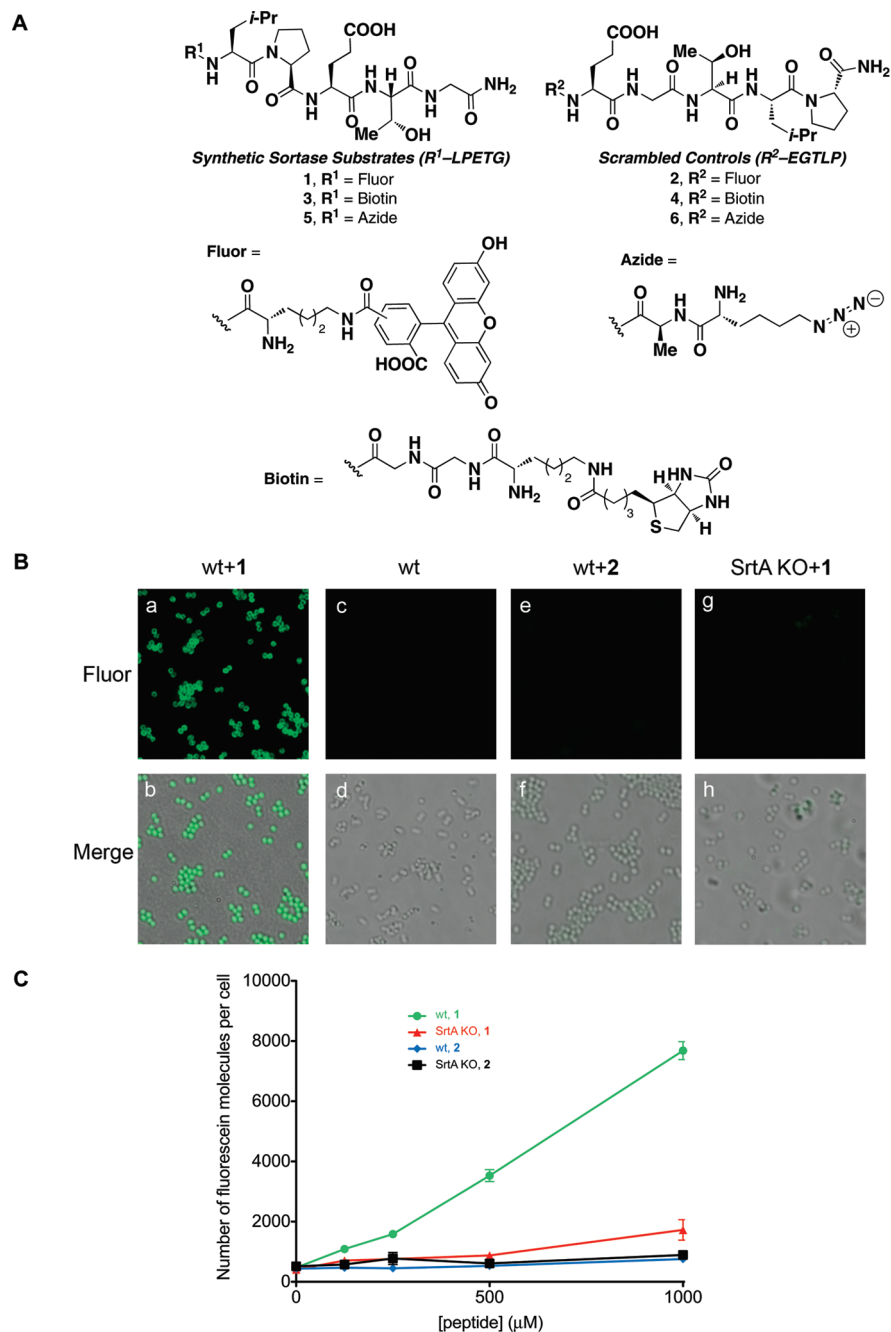
A

Synthetic Sortase Substrates (R¹–LPETG)
1, R¹ = Fluor
3, R¹ = Biotin
5, R¹ = Azide

Scrambled Controls (R²–EGTLP)
2, R² = Fluor
4, R² = Biotin
6, R² = Azide

Fluor =

Azide =

Biotin =

B

C

Figure 2. Observation and measurement of incorporated fluorescence into wild-type or SrtA-knockout *S. aureus* following small molecule treatment. A) Synthetic small molecules employed in these studies. B) Epifluorescence microscopy. Abbreviations: "wt" indicates wild-type *S. aureus*, "SrtA KO" indicates a sortase A knockout strain of *S. aureus* (*16*), "Fluor" indicates epifluorescence images, and "Merge" corresponds to fluorescent images superimposed atop brightfield images. All micrographs were acquired under identical exposure settings and processed in an identical fashion. The depicted trends were reproduced on at least three separate occasions. C) Quantitation of fluorescein incorporation *via* flow cytometry.

A.

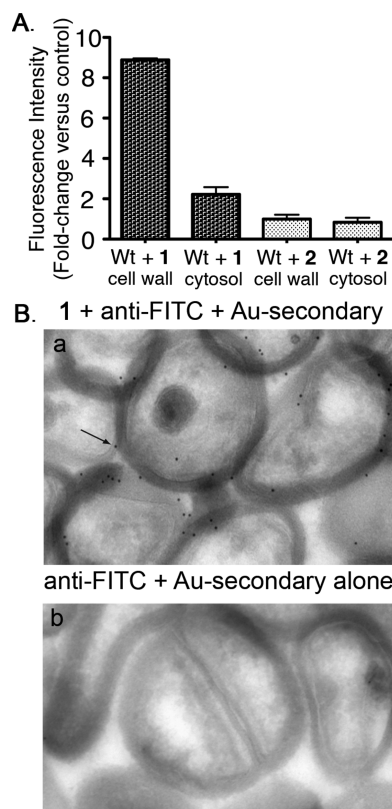**B.** 1 + anti-FITC + Au-secondary

a

anti-FITC + Au-secondary alone

b

Figure 3. SrtA-targeted small molecules are localized to the cell wall. A) Fractionation of wild-type *S. aureus* cells into cell wall and cytosolic extracts following treatment with compounds 1 or 2. Data represent the average of triplicate experiments ± SD. B) Immunocryoelectron microscopy studies of a protein A-knockout *S. aureus* strain in the Newman genetic background. Cells were incubated in the presence (panel a) and absence (panel b) of 1. Arrow points to an example of black dots, which correspond to gold-labeled antibody binding. Abbreviations: anti-FITC = rabbit anti-fluorescein antibody; Au-secondary = gold-labeled anti-rabbit secondary antibody.

incubated with **1** exhibited approximately 8-fold greater fluorescence *versus* those isolated from organisms treated with scrambled peptide **2**. Further, a 3.5-fold increase in the fluorescence of cell wall *versus* cytosolic fractions following treatment with **1** was observed. Interestingly, **1**-treated bacteria exhibited a small increase in fluorescence compared to the control, indicating that

fluorescein-LPETG constructs but not scrambled sequences partition into cytosolic extracts. This may result from a number of processes including contamination of cytosolic extracts with membrane vesicles containing peptide bound to SrtA but not yet incorporated into cell wall, interactions with other membrane-associated enzymes (such as *S. aureus* LPXTGase) (*18, 19*), or recycling of peptidoglycan components into the cytosol for reuse (*20*).

Localization of **1** to the peptidoglycan layer was confirmed *via* immunocryoelectron microscopy (Figure 3B, panels a and b). Here a *S. aureus* mutant deficient in cell wall protein A (*21*) was incubated with peptide **1** and then subjected to thin sectioning and immunostaining with an anti-fluorescein antibody followed by a gold-conjugated secondary antibody. Use of a protein A-knockout was necessary here to prevent background labeling of cell wall through interactions between protein A and the antibody's Fc region. As shown, >90% of gold labeling was observed in the vicinity of the cell wall. A small amount of apparent intracellular labeling was also detected, perhaps due to antibody background or to the presence of overlapping cells in thin sections; some degree of intracellular uptake or recycling of compound **1** cannot be ruled out.

Measuring Covalency of SrtA-Targeted Small Molecules. To demonstrate unambiguously that small molecules were covalently incorporated into the cell wall, we utilized a MALDI-TOF MS-based strategy (*22*). These experiments provide direct evidence for covalent attachment of synthetic small molecules into the Gram-positive bacterial cell wall. *S. aureus* cultures treated with and without constructs **3** and **4** were first digested with the enzymes mutanolysin and lysostaphin to degrade the cell wall and release cell wall associated proteins. Digests were then subjected to streptavidin affinity chromatography followed by mass spectrometric analysis. Because lysostaphin is a *Gly-Gly* endopeptidase and *S. aureus* cell wall proteins are tethered to the peptidoglycan through a pentaglycine cross-link, we anticipated that processing of **3**-treated bacteria in our mass spectrometry protocol would provide a biotin-LPET fragment attached to multiple glycine residues (between 1 and 4).

Indeed, extracts from cells treated with **3** and analyzed through the above sequence exhibited strong peak enrichments at 1098.60 and 1120.60 amu, which were absent from all control conditions (Figure 4). These
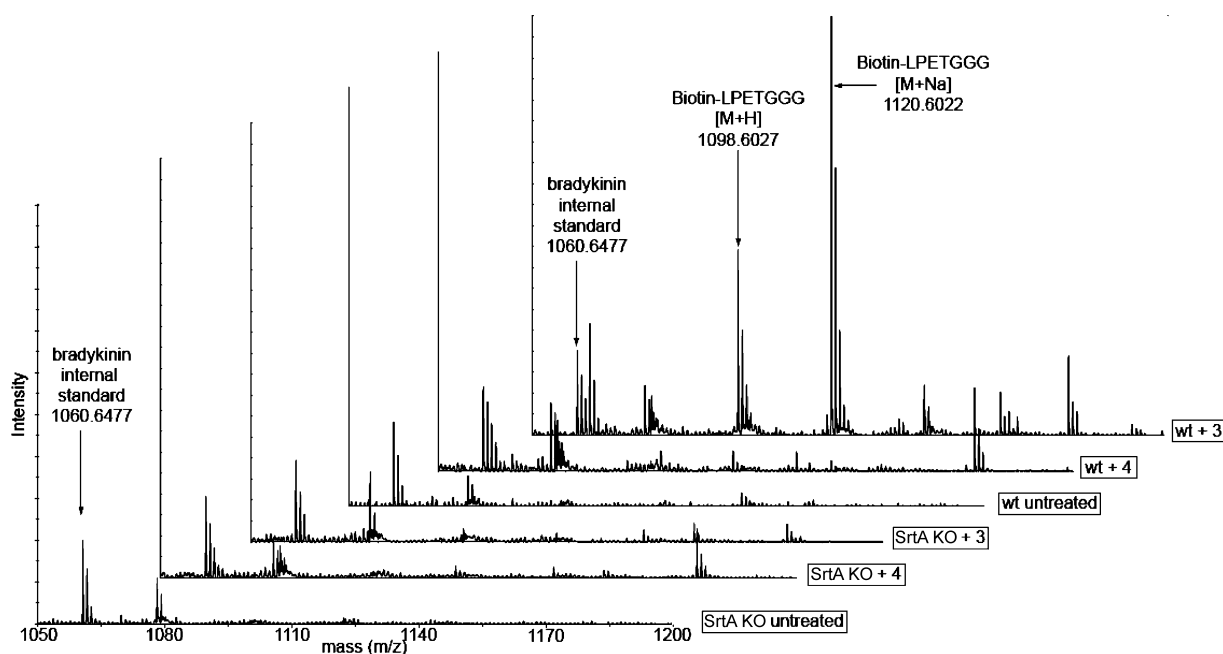
Figure 4. MALDI-TOF mass spectra of streptavidin affinity-purified *S. aureus* cell wall extracts after treatment of both wild-type and SrtA-knockout strains with and without biotin-peptide hybrids 3 **and** 4. **Abbreviations: "wt" indicates wild-type *S. aureus*, and "SrtA KO" indicates a sortase A knockout strain of *S. aureus* (***16***).**

masses correspond to [M + H] and [M + Na] ions for the biotin-LPETGGG sequence, whose identity we were able to confirm *via* MALDI MS/MS sequencing (Supplementary Figure 3). Masses corresponding to biotin-LPETGG and biotin-LPETGGGG were not well resolved in these spectra, which is consistent with observations from previous mass spectrometry studies of cell wall proteins released from *S. aureus* following lysostaphin digestion (*23*). Taken together, these data provide strong evidence supporting covalent attachment of our synthetic materials into the *S. aureus* cell wall through a mechanism involving SrtA.

Bioorthogonal [3 + 2] Cycloaddition on the *S. aureus* Surface. Bioorthogonal chemical reactions have proven useful in the study of a wide array of biological processes (*24, 25*). Only a few studies, however, have employed bioorthogonal reactions in bacteria (*26–32*). Furthermore, none of these to our knowledge have been executed in S. *aureus*. Accordingly, we synthesized molecules **5** and **6**, which both contain N$^\varepsilon$-azido-L-lysine residues at their N-termini (Figure 2A). The azide functionality in **5** and **6** was introduced because of its ability to undergo a [3 + 2] cycloaddition ("click") reaction

with alkyne-containing probes (*33, 34*). To this end, we prepared Alexa Fluor 488-conjugated cyclooctyne probe **7** (Figure 5A). Structural analogues of compound **7** have been shown to participate efficiently in bioorthogonal copper-free click chemistry (*24, 35–38*). Wild-type *S. aureus* cultured in the presence of putative SrtA substrate **5** and then treated with compound **7** demonstrated a marked increase in fluorescence *versus* both background and scrambled control **6**, as determined by flow cytometry and epifluorescence microscopy (Figure 5). These results demonstrate the ability of non-natural SrtA substrates to decorate the staphylococcal cell wall with reactive groups and represent the first bioorthogonal chemical reaction performed on the surface of *S. aureus*.

Conclusions. Strategies for re-engineering cell surfaces with small molecules have emerged as powerful tools to perturb and study biological processes both *in vitro* and *in vivo* (*39, 40*). Despite this, relatively few such approaches involving bacteria have appeared (*26–32, 41, 42*). Here we demonstrate for the first time that the *S. aureus* surface can be modified to incorporate exogenous, non-native small molecules by way of
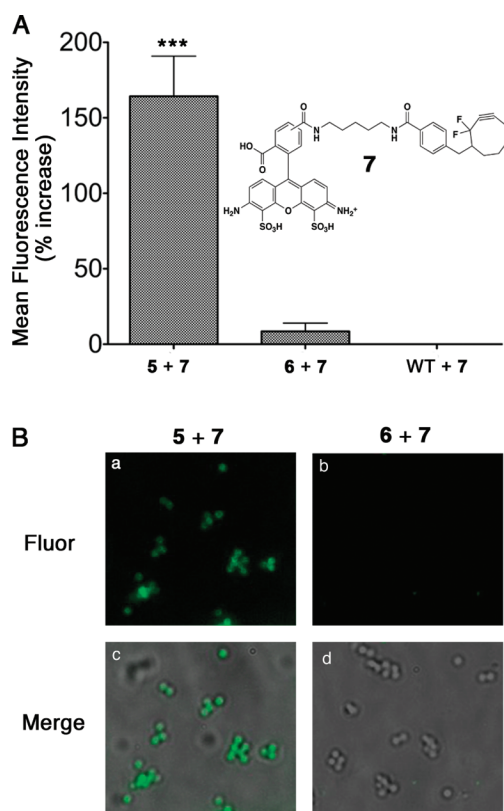
Figure 5. A) Flow cytometry-based analysis of wild-type *S. aureus* labeled with 5 or 6 following reaction with compound 7. Data are reported as percent increase versus control (wild-type bacteria incubated with 7 without pretreatment with 5 or 6), and represent the mean of three replicate experiments ± SD. Data in the left-hand column were found to exhibit a highly statistically significant increase in fluorescence (***, $P <$ 0.0001) compared to that in the middle or right-hand columns following a one-way analysis of variance (ANOVA) with Tukey's multiple comparison posthoc test. **B)** Epifluorescence microscopy. "Fluor" indicates epifluorescence images, and "Merge" corresponds to fluorescent images superimposed atop brightfield images. All micrographs were acquired under identical exposure settings and processed in an identical fashion. The depicted trends were reproduced on at least two separate occasions.

its own biosynthetic machinery. Epifluorescence microscopy and flow cytometry experiments demonstrate that these molecules are incorporated into bacteria at high levels in a SrtA- and LPETG-dependent fashion. Subsequent cell wall extraction and electron microscopy data indicate that these non-native materials are localized to the cell wall, and mass spectrometry data confirms that this labeling phenomenon involves covalent bond formation. Finally, we exploit this biosynthetic labeling technology to perform a bioorthogonal chemical reaction on the *S. aureus* surface.

A diverse range of applications of this strategy can be envisioned. For example, SrtA-mediated biosynthetic incorporation of fluorescent materials into bacterial peptidoglycan could enable the development of novel *in situ* imaging protocols. Because labeling is SrtA-dependent and highly selective and does not affect bacterial viability, it should prove advantageous in comparison to conventional techniques for labeling bacteria with small molecules. Such strategies often involve promiscuous electrophiles (*e.g.*, FITC) and can perturb bacterial viability and/or behavior (*43*). The reported strategy might also be useful in developing engineered organisms with entirely novel functions. Indeed, by enabling the display of chemical functionality that cannot yet be genetically encoded, SrtA-mediated biosynthetic incorporation processes could complement ongoing efforts in synthetic biology, which rely predominantly on genetic manipulations (*44*). Given that SrtA is widely distributed among Gram-positive bacteria, this strategy could be applied in diverse species including *Streptococcus pneumoniae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, and many others. Also, because sortase enzymes in species other than *S. aureus* often possess unique recognition and anchoring elements (other than LPXTG) (*45*), this strategy may prove highly selective, even in complex settings. Finally, biosynthetic incorporation of small molecules into bacteria could lead to novel therapeutic approaches. Strategies that employ small molecules to enhance immunological recognition of pathogens have proven quite promising (*46–51*); however, to our knowledge, none of these have utilized biosynthetic mechanisms to incorporate immunotargeting moieties. Such technologies could possess unique advantages over available antibiotic modalities and lead to novel paradigms in the treatment of patients suffering from bacterial illness.

METHODS

Cell Culture and Reagents. Wild-type *S. aureus* Newmann and a matching SrtA− strain were kindly donated by O. Schneewind (University of Chicago) (*16*). *S. aureus* strain *spa::kan* (*21*), a protein A knockout strain, was obtained from T. J. Foster (Trinity College, Dublin, Ireland).

Peptide Synthesis Procedure. Rink amide resin preloaded with either N^α-Fmoc-Gly or N^α-Fmoc-Pro was mixed with four column volumes (CV) of dimethylformamide (DMF) for 1 h prior to the commencement of each peptide synthesis. Deprotection of the resin prior to coupling was accomplished on shaking in 4 CV of 20% piperidine/DMF for 30 min. For all subsequent coupling reactions, Fmoc-protected amino acid (5 equiv), *O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, 5 equiv), and *i*-Pr$_2$NEt (10 equiv) were suspended with the resin in DMF (4 CV) and shaken at RT for 4 h. In all syntheses, Fmoc-protecting groups were removed by shaking for 5 min in 50% piperidine/DMF (4 CV).

For peptide cleavage reactions, resin was first treated with a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water (94:3:3, 10 mL) at RT for 90 min. Cleavage solutions were then filtered through cotton into cold ether, pelleted, resuspended twice in ether, and dried *in vacuo*. Peptides were purified *via* high pressure liquid chromatography (HPLC) and lyophilized, and their identities were confirmed *via* high-resolution mass spectrometry.

Synthesis of Alexa Fluor 488-DIFO Conjugates (*7*). Perfluorophenyl 4-((2,2-difluorocyclooct-3-ynyl)methyl)benzoate (3.1 mg, 7 μmol, 2 equiv) and *i*-Pr$_2$NEt (2.4 μL, 14 μmol, 4 equiv) were dissolved in DMF (0.5 mL) followed by Alexa Fluor 488 cadaverine (2 mg, 3.1 μmol, 1 equiv), whose transfer was aided by the use of DMF (0.5 mL). The reaction mixture was stirred for 48 h, concentrated, and purified by HPLC to give **7**. Suspension in 2 mL of PBS led to a 200 μM solution (0.4 μmol, 13% yield) as determined by UV absorbance at 495 nm.

Generic Bacterial Labeling Procedure. Bacteria were grown for 24 h in Luria Broth at 37 °C (supplemented with 10 μg mL^{-1} erythromycin for sortase-deficient mutants) containing compounds at the desired concentration (1 mM unless otherwise noted, final volume 500 μL). Subsequently, 100 μL aliquots of culture were taken, pelleted at $12,000 \times g$ for 1 min, and washed three times with PBS buffer. Cells were then used in the following experiments.

Microscopy/Flow Cytometry. Labeled cells were resuspended in 4% paraformaldehyde in PBS and incubated at RT on a nutating mixer for 45 min. Following three additional PBS washes, bacteria were resuspended in 1 mL of PBS and analyzed on an Accuri C6 Flow Cytomer. Levels of fluorescein incorporation were quantitated by comparison to Quantum FITC MESF beads (Bangs Laboratories). Aliquots (100 μL) of the above were removed (prior to cytometric analysis), pelleted, and resuspended in 20 μL of anti-fade media (Vectashield Mounting Media), mounted onto Esco Superfrost Microscope slides, and allowed to incubate overnight at 4 °C before images were obtained.

Quantitation of Fluorescence *via* Cell Wall Extraction. Following labeling, bacteria were diluted with PBS to normalize total bacterial count and subjected to ultrasonication. The sonicated cells were filtered *via* centrifugal filtration using Amicon YM-10 filters at 4 °C for 30 min. The filtrate was taken and used to determine the fluorescence intensity of the cytosol fraction, while the high-molecular-weight pellet was collected and treated for 30 min at 37 °C with 500 μL of SMM buffer containing 50 μg mL^{-1} lysostaphin prewarmed to 37 °C. The mixture was then centrifuged at 14,000 rpm for 1 min, and the supernatant was removed and placed on ice. Next, 500 μL of 10% sodium dodecyl sulfate (SDS) was added to the cold supernatant mixtures, 100 μL of each sample was taken, and fluorescence intensities

were determined in triplicate using a Tecan Platereader (em 485 nm, ex 535 nm). Gain was set to the intensity of the wild-type bacterial cell wall that had been incubated with peptide. Cell wall fractionation data were derived by first subtracting the appropriate blank (cell wall or cytosol) from fluorescence intensity values and then measuring the difference between this value and the fluorescence reading obtained for control conditions (*i.e.*, wild-type bacteria not treated with peptide). Entries represent means from triplicate experiments, and error bars indicate standard deviations.

Electron Microscopy. Following the generic protocol for bacterial labeling outlined above, bacteria were resuspended in 4% paraformaldehyde in PBS and incubated at RT on a nutating mixer for 45 min. Samples were then pelleted, washed three additional times with PBS, and suspended in 5% gelatin. Suspensions were trimmed to small blocks and placed in 2.3 M sucrose solution (cryoprotectant) overnight on a rotor at 40 °C. They were transferred to aluminum pins and frozen rapidly in liquid nitrogen. The frozen block was trimmed on a Leica CryoUltraCut, and 75 nm thick sections were collected using the Tokoyasu method (*52*). The frozen sections were collected on a drop of sucrose, thawed, placed on a nickel Formvar/carbon coated grid, and floated in a dish of PBS ready for immunolabeling.

For immunolabeling, grids were placed on drops of 0.1 M ammonium chloride for 10 min to quench untreated aldehyde groups and then blocked for non-specific binding on 1% fish skin gelatin for 20 min. Grids were incubated on primary antibody (rabbit anti-FITC IgG, Invitrogen) at 1:10 and 1:50 dilutions for 30 min. Controls were also performed using Rabbit IgG (Jackson) at the same dilution. Samples were then rinsed 6 times on drops of PBS and labeled using 12 nm anti-rabbit colloidal gold (Jackson) for 30 min. Grids were rinsed in PBS and fixed using 1% gluteraldehyde for 5 min, and a final rinse step with distilled water was performed before transferring grids to a UA/methylcellulose drop for 10 min. Grids were dried and viewed in a FEI Tencai Biotwin TEM at 80 Kv. Images were acquired using Morada CCD and iTEM (Olympus) software.

MALDI-TOF Mass Spectrometry. Following the generic protocol for labeling outlined above, bacteria were diluted with 3.5 mL of PBS and subjected to ultrasonication. The pellet was then subjected to enzymatic digest with mutanolysin (150 μg mL^{-1}) and lysostaphin (100 μg mL^{-1}) in 500 μL of Tris-HCl buffer (0.5 mM, pH 6.5). After gentle mixing, the solution was incubated at 37 °C for 4 h. Samples were then pelleted and the supernatant heated at 90 °C for 5 min before isolation of biotinylated adducts.

Biotinylated peptides were isolated using an Avidin cartridge (ABI) with the ABI syringe setup and ABI protocols. The avidin eluted biotinylated peptides were dried in a Savant SVC100 SpeedVac, prior to dissolving in 4 μL of matrix solvent (0.1% trifluoroacetic acid, 50% acetonitrile/water). Matrix was prepared by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in the matrix solvent to a concentration of 3.0 mg mL^{-1}. Bradykinin was added to the matrix as an internal calibrant at a final concentration of 1.67 fmol μL^{-1}. Spotting mixtures were prepared by mixing 1 μL of sample with 4 μL of matrix in an Eppendorf tube. Then, 0.8 μL of each mixture was spotted on a 384-well sample target plate and allowed to air-dry. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) was acquired on an Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF Analyzer in reflector positive mode. The mass range from 700−4000 *m/z* was screened, and the spectra were internally calibrated to the bradykinin standard (protonated, monoisotopic mass of 1060.569 Da).

Cell-Surface Labeling with DIFO Reagents. Following the generic protocol for labeling outlined above, bacteria were resuspended in ice-cold PBS (500 μL). Click-reagent **7** was then

added to the suspended bacteria to a final concentration of 10 μM, and cultures were then incubated for 48 h at 4 °C on a nutating mixer. Cells were washed three times with PBS and then fixed with 4% PFA for 30 min. Following three more washes, the bacteria were resuspended in 1 mL of PBS and analyzed on an Accuri C6 Flow Cytometer.

For microscopy, 100 μL aliquots of the fixed samples from above were removed prior to cytometric analysis. The aliquots were pelleted by centrifugation, resuspended in 5 μL of Fluoro-Gel, mounted on Esco Superfrost Microscope slides, and allowed to incubate overnight at 4 °C before images were obtained.

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Taubes, G. (2008) The bacteria fight back, *Science 321*, 356–361.
2. Klevens, R., Morrison, M., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L., Lynfield, R., Dumyati, G., Townes, J., Craig, A., Zell, E., Fosheim, G., Mcdougal, L., Carey, R., and Fridkin, S. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States, *J. Am. Med. Assoc. 298*, 1763–1771.
3. Center for Disease Control, HIV/AIDS Surveillance Report: HIV Infection and AIDS in the United States and Dependent Areas,, (2006) . Available at http://www.cdc.gov/hiv/topics/surveillance/resources/reports/2006report/table7.htm.
4. Rooijakkers, S. H., van Kessel, K. P., and van Strijp, J. A. (2005) Staphylococcal innate immune evasion, *Trends Microbiol. 13*, 596–601.
5. Serruto, D., Rappuoli, R., Scarselli, M., Gros, P., and van Strijp, J. A. (2010) Molecular mechanisms of complement evasion: learning from staphylococci and meningococci, *Nat. Rev. Microbiol. 8*, 393–399.
6. Maresso, A., and Schneewind, O. (2008) Sortase as a target of anti-infective therapy, *Pharmacol. Rev. 60*, 128–141.
7. Parthasarathy, R., Subramanian, S., and Boder, E. T. (2007) Sortase A as a novel molecular "stapler" for sequence-specific protein conjugation, *Bioconjugate Chem. 18*, 469–476.
8. Antos, J. M., Miller, G. M., Grotenbreg, G. M., and Ploegh, H. L. (2008) Lipid modification of proteins through sortase-catalyzed transpeptidation, *J. Am. Chem. Soc. 130*, 16338–16343.
9. Guo, X., Wang, Q., Swarts, B. M., and Guo, Z. (2009) Sortase-catalyzed peptide-glycosylphosphatidylinositol analogue ligation, *J. Am. Chem. Soc. 131*, 9878–9879.
10. Pritz, S., Wolf, Y., Kraetke, O., Klose, J., Bienert, M., and Beyermann, M. (2007) Synthesis of biologically active peptide nucleic acid-peptide conjugates by sortase-mediated ligation, *J. Org. Chem. 72*, 3909–3912.
11. Wu, Z., Guo, X., Wang, Q., Swarts, B. M., and Guo, Z.Sortase A-catalyzed transpeptidation of glycosylphosphatidylinositol derivatives for chemoenzymatic synthesis of GPI-anchored proteins, *J. Am. Chem. Soc. 132*, 1567–1571.
12. Popp, M. W., Antos, J. M., Grotenbreg, G. M., Spooner, E., and Ploegh, H. L. (2007) Sortagging: a versatile method for protein labeling, *Nat. Chem. Biol. 3*, 707–708.
13. Yamamoto, T., and Nagamune, T. (2009) Expansion of the sortase-mediated labeling method for site-specific N-terminal labeling of cell surface proteins on living cells, *Chem. Commun.* 1022–1024.
14. Tanaka, T., Yamamoto, T., Tsukiji, S., and Nagamune, T. (2008) Site-specific protein modification on living cells catalyzed by Sortase, *ChemBioChem 9*, 802–807.
15. Popp, M. W., Antos, J. M., Ploegh, H. L. (2009) Site-specific protein labeling via sortase-mediated transpeptidation, *Curr. Protoc. Protein. Sci.* Suppl. 56, Unit 15.13.
16. Mazmanian, S. K., Liu, G., Jensen, E. R., Lenoy, E., and Schneewind, O. (2000) *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections, *Proc. Natl. Acad. Sci. U.S.A. 97*, 5510–5515.
17. Mohamed, N., Visai, L., Speziale, P., and Ross, J. M. (2000) Quantification of *Staphylococcus aureus* cell surface adhesins using flow cytometry, *Microb. Pathog. 29*, 357–361.
18. Lee, S. G., and Fischetti, V. A. (2006) Purification and characterization of LPXTGase from *Staphylococcus aureus*: the amino acid composition mirrors that found in the peptidoglycan, *J. Bacteriol. 188*, 389–398.
19. Lee, S. G., Pancholi, V., and Fischetti, V. A. (2002) Characterization of a unique glycosylated anchor endopeptidase that cleaves the LPXTG sequence motif of cell surface proteins of Gram-positive bacteria, *J. Biol. Chem. 277*, 46912–46922.
20. Litzinger, S., Duckworth, A., Nitzsche, K., Risinger, C., Wittmann, V., and Mayer, C. (2010) Muropeptide rescue in *Bacillus subtilis* involves sequential hydrolysis by β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-ʟ-alanine amidase, *J. Bacteriol. 192*, 3132–3143.
21. Patel, A. H., Foster, T. J., and Pattee, P. A. (1989) Physical and genetic mapping of the Protein A gene in the chromosome of *Staphylococcus aureus* 8325-4, *J. Gen. Microbiol. 135*, 1799–1807.
22. Ton-That, H., Faull, K. F., and Schneewind, O. (1997) Anchor structure of staphylococcal surface proteins. A branched peptide that links the carboxyl terminus of proteins to the cell wall, *J. Biol. Chem. 272*, 22285–22292.
23. Schneewind, O., Fowler, A., and Faull, K. F. (1995) Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*, *Science 268*, 103–106.
24. Sletten, E. M., and Bertozzi, C. R. (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality, *Angew. Chem., Int. Ed. 48*, 6974–6998.
25. Best, M. D. (2009) Click chemistry and bioorthogonal reactions: unprecedented selectivity in the labeling of biological molecules, *Biochemistry 48*, 6571–6584.
26. Yi, W., Liu, X., Li, Y., Li, J., Xia, C., Zhou, G., Zhang, W., Zhao, W., Chen, X., and Wang, P. G. (2009) Remodeling bacterial polysaccharides by metabolic pathway engineering, *Proc. Natl. Acad. Sci. U.S.A. 106*, 4207–4212.
27. Sadamoto, R., Niikura, K., Ueda, T., Monde, K., Fukuhara, N., and Nishimura, S. I. (2004) Control of bacteria adhesion by cell-wall engineering, *J. Am. Chem. Soc. 126*, 3755–3761.
28. Sadamoto, R., Niikura, K., Sears, P. S., Liu, H. T., Wong, C. H., Suksomcheep, A., Tomita, F., Monde, K., and Nishimura, S. I. (2002) Cell-wall engineering of living bacteria, *J. Am. Chem. Soc. 124*, 9018–9019.
29. Sadamoto, R., Niikura, K., Monde, K., and Nishimura, S. I. (2003) Cell wall engineering of living bacteria through biosynthesis, *Methods Enzymol. 362*, 273–286.
30. Sadamoto, R. (2005) Artificial modification of bacterial surface with cell-wall engineering, *Trends Glycosci. Glycotechnol. 17*, 97–105.
31. Link, A. J., Vink, M. K., and Tirrell, D. A. (2004) Presentation and detection of azide functionality in bacterial cell surface proteins, *J. Am. Chem. Soc. 126*, 10598–10602.

32. Beatty, K. E., Xie, F., Wang, Q., and Tirrell, D. A. (2005) Selective dye-labeling of newly synthesized proteins in bacterial cells, *J. Am. Chem. Soc. 127*, 14150–14151.

33. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, *Angew. Chem., Int. Ed. 41*, 2596–2599.

34. Tornøe, C., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, *J. Org. Chem. 67*, 3057–3064.

35. Codelli, J. A., Baskin, J. M., Agard, N. J., and Bertozzi, C. R. (2008) Second-generation difluorinated cyclooctynes for copper-free click chemistry, *J. Am. Chem. Soc. 130*, 11486–11493.

36. Chang, P. V., Prescher, J. A., Hangauer, M. J., and Bertozzi, C. (2007) Imaging cell surface glycans with bioorthogonal chemical reporters, *J. Am. Chem. Soc. 129*, 8400–8401.

37. Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. (2008) *In vivo* imaging of membrane-associated glycans in developing zebrafish, *Science 320*, 664–667.

38. Prescher, J. A., and Bertozzi, C. R. (2005) Chemistry in living systems, *Nat. Chem. Biol. 1*, 13–21.

39. Chang, P. V., Prescher, J. A., Sletten, E. M., Baskin, J. M., Miller, I. A., Agard, N. J., Lo, A., and Bertozzi, C. R. (2010) Copper-free click chemistry in living animals, *Proc. Natl. Acad. Sci. U.S.A. 107*, 1821–1826 and references therein.

40. Agard, N. J., and Bertozzi, C. R. (2009) Chemical approaches to perturb, profile, and perceive glycans, *Acc. Chem. Res. 42*, 788–797.

41. Schilling, B., Goon, S., Samuels, N. M., Gaucher, S. P., Leary, J. A., Bertozzi, C. R., and Gibson, B. W. (2001) Biosynthesis of sialylated lipooligosaccharides in *Haemophilis ducreyi* is dependent on exogenous sialic acid and not mannosamine. Incorporation studies using *N*-acylmannosamine analogues, *N*-glycolylneuraminic acid, and C-13-labeled *N*-acetylneuraminic acid, *Biochemistry 40*, 12666–12677.

42. Goon, S., Schilling, B., Tullius, M. V., Gibson, B. W., and Bertozzi, C. R. (2003) Metabolic incorporation of unnatural sialic acids into *Haemophilus ducreyi* lipooligosaccharides, *Proc. Natl. Acad. Sci. U.S.A. 100*, 3089–3094.

43. Weingart, C. L., Broitman-Maduro, G., Dean, G., Newman, S., Peppler, M., and Weiss, A. A. (1999) Fluorescent labels influence phagocytosis of *Bordetella pertussis* by human neutrophils, *Infect. Immun. 67*, 4264–4267.

44. Agapakis, C. M., and Silver, P. A. (2009) Synthetic biology: exploring and exploiting genetic modularity through the design of novel biological networks, *Mol. Biosyst. 5*, 704–713.

45. Boekhorst, J., de Been, M. W. H. J., Kleerebezem, M., and Siezen, R. J. (2005) Genome-wide detection and analysis of cell wall-bound proteins with LPxTG-Like sorting motifs, *J. Bacteriol. 187*, 4928–4934.

46. Carlson, C. B., Mowery, P., Owen, R. M., Dykhuizen, E. C., and Kiessling, L. L. (2007) Selective tumor cell targeting using low-affinity, multivalent interactions, *ACS Chem. Biol. 2*, 119–127 and references therein.

47. Low, P., Henne, W., and Doorneweerd, D. (2008) Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases, *Acc. Chem. Res. 41*, 120–129 and references therein.

48. Murelli, R. P., Zhang, A. X., Michel, J., Jorgensen, W. L., and Spiegel, D. A. (2009) Chemical control over immune recognition: a class of antibody-recruiting small molecules that target prostate cancer, *J. Am. Chem. Soc. 131* (47), 17090–17092 and references therein.

49. Parker, C. G., Domaoal, R. A., Anderson, K. S., and Spiegel, D. A. (2009) An antibody-recruiting small molecule that targets HIV gp120, *J. Am. Chem. Soc. 131*, 16392–16394 and references therein.

50. Popkov, M., Gonzalez, B., Sinha, S. V., and Barbas, C. F., III (2009) Instant immunity through chemically programmable vaccination and covalent self-assembly, *Proc. Natl. Acad. Sci. U.S.A. 106*, 4378–4383 and references therein.

51. Krishnamurthy, V. M., Quinton, L. J., Estroff, L. A., Metallo, S. J., Isaacs, J. M., Mizgerd, J. P., and Whitesides, G. M. (2006) Promotion of opsonization by antibodies and phagocytosis of Gram-positive bacteria by a bifunctional polyacrylamide, *Biomaterials 27*, 3663–3674.

52. Tokuyasu, K. T. (1973) A technique for ultracryotomy of cell suspensions and tissues, *J. Cell Biol. 57*, 551–565.