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Discovery of a Small Molecule that Blocks Wall Teichoic Acid Biosynthesis in *Staphylococcus aureus*

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ABSTRACT Both Gram-positive and Gram-negative bacteria contain bactoprenol-dependent biosynthetic pathways expressing non-essential cell surface polysaccharides that function as virulence factors. Although these polymers are not required for bacterial viability *in vitro*, genes in many of the biosynthetic pathways are conditionally essential: they cannot be deleted except in strains incapable of initiating polymer synthesis. We report a cell-based, pathway-specific strategy to screen for small molecule inhibitors of conditionally essential enzymes. The screen identifies molecules that prevent the growth of a wildtype bacterial strain but do not affect the growth of a mutant strain incapable of initiating polymer synthesis. We have applied this approach to discover inhibitors of wall teichoic acid (WTA) biosynthesis in *Staphylococcus aureus*. WTAs are anionic cell surface polysaccharides required for host colonization that have been suggested as targets for new antimicrobials. We have identified a small molecule, 7-chloro-*N*,*N*-diethyl-3-(phenylsulfonyl)-[1,2,3]triazolo[1,5-*a*]quinolin-5-amine (1835F03), that inhibits the growth of a panel of *S. aureus* strains (MIC = 1−3 μg mL^{-1}), including clinical methicillin-resistant *S. aureus* (MRSA) isolates. Using a combination of biochemistry and genetics, we have identified the molecular target as TarG, the transmembrane component of the ABC transporter that exports WTAs to the cell surface. We also show that preventing the completion of WTA biosynthesis once it has been initiated triggers growth arrest. The discovery of 1835F03 validates our chemical genetics strategy for identifying inhibitors of conditionally essential enzymes, and the strategy should be applicable to many other bactoprenol-dependent biosynthetic pathways in the pursuit of novel antibacterials and probes of bacterial stress responses.

Antibiotic-resistant bacterial infections pose a major threat to public health (*1*). In recent years, increasing effort has been devoted to exploring unconventional antibacterial targets, including virulence factors (*2*). Virulence factors are bacterially expressed molecules that are non-essential for survival *in vitro* but are required for robust infection in a host. Small molecule inhibitors have been discovered for many virulence factors, including the type III secretion machinery, two-component signaling and quorum sensing systems, and cholera toxin (*3, 4*). A number of studies suggest that antivirulence factor inhibitors hold promise for limiting bacterial infections (*5*–*7*).

The cell envelope of many pathogenic bacteria contains a large and diverse group of polysaccharide polymers that function as virulence factors in that they are non-essential *in vitro* but required for pathogenicity. Although many of these polymers are potential targets for new antibacterials, the discovery of inhibitors that block their synthesis has been hampered by difficulties in reconstituting the enzymatic machinery, obtaining lipid-linked substrates, and developing high-throughput assays. A whole cell assay would circumvent some of these challenges *provided* it could be designed to report specifically on inhibition of the targeted pathway.

Many biosynthetic pathways for cell surface polymers share a common feature that suggests a strategy for inhibitor discovery: they contain conditionally essential genes. That is, the initiating genes, the products of which are responsible for the first steps of polymer synthesis, are dispensable since the polymers are non-essential *in vitro*, but the downstream genes are essential *unless* polymer initiation is prevented. Stably

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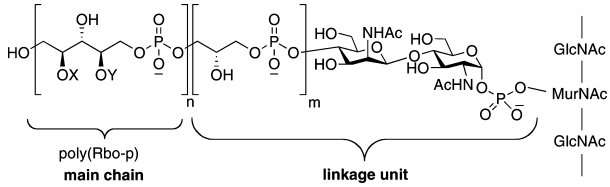

Figure 1. Chemical structure of WTAs in *Staphylococcus aureus*. Rbo-p = ribitol-phosphate; *m* = 1–3; *n* = 20–40; X = D-alanine or H; Y = α- or β-GlcNAc (*20*).

maintained gene deletions of conditionally essential enzymes are thus generally accompanied by suppressor mutations that prevent committed flux into these biosynthetic pathways (*8–13*). The conditional essentiality of genes in non-essential, bactoprenol-dependent biosynthetic pathways was first observed in 1969 for O-antigen biosynthesis in *Salmonella enterica* (*8*) and has since been extended to capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* (*9*), exopolysaccharide biosynthesis in the plant pathogen *Xanthomonas campestris* (*10*), O-antigen biosynthesis in *Escherichia coli* and *Pseudomonas aeruginosa* (*11, 12*), and wall teichoic acid (WTA) biosynthesis in *S. aureus* (*13*). As with peptidoglycan biosynthesis, these polysaccharides are all assembled intracellularly on a bactoprenol carrier lipid before transfer to the final acceptor on the surface of the cell (*14*). It has been suggested that blocking flux once polymer synthesis has been initiated is deleterious either because toxic bactoprenol-linked polysaccharide intermediates accumulate or because bactoprenol-linked peptidoglycan precursors are depleted.

We demonstrate the utility of a general screening strategy for inhibitors of conditionally essential enzymes by discovering an inhibitor of WTA biosynthesis in the clinically relevant pathogen *Staphylococcus aureus*. Methicillin-resistant *S. aureus* (MRSA) infections have become a major problem both in hospitals and in the community (*15*). Further, high level vancomycin resistance in MRSA has begun to appear, creating a clear demand for antimicrobials against new targets. The WTA biosynthetic pathway is a possible target since *S. aureus* strains lacking WTAs are unable to colonize host tissue and exhibit a greatly diminished capacity to establish infections in animal models (*16–18*). No specific inhibitors of WTA biosynthesis have been previously identified, although a compound that prevents D-alanylation, a tailoring modification of both WTAs and a related family of anionic cell surface polymers, lipoteichoic acids, has been shown to affect bacterial fitness (*19*).

WTAs from *S. aureus* are disaccharide-linked anionic polymers of poly(ribitol-phosphate) that are covalently attached to peptidoglycan (Figure 1). They constitute a large percentage of the total cell wall mass (*20*). The primary *S. aureus* WTA is assembled on a bactoprenol carrier embedded in the cytoplasmic membrane by the sequential addition of two sugar residues (by TarO and TarA), two to three glycerol 3-phosphate units (by TarB and TarF), and then finally the poly(ribitol-phosphate) repeat (by TarL) (Figure 2) (*21, 22*). WTAs are then exported through the membrane by an ABC (ATP binding cassette) transporter complex (TarGH), and the polymer is transferred from the bactoprenol carrier to peptidoglycan by an unidentified transferase (*20*).

As found in several other bactoprenol-dependent biosynthetic pathways, the first gene in the WTA pathway (*tarO*) can be deleted since the pathway is non-essential (*13, 16*). Similarly, the second gene, *tarA*, can also be deleted since the initiating enzyme (TarO) is readily reversible, as are other phosphosugar transferases (*23*). However, the downstream genes (*tarBDFGHIJL*) can only be deleted in a strain that cannot initiate polymer synthesis (Figure 2) (*13*). Therefore, we devised a high-throughput screen that involved comparing the growth of a wildtype *S. aureus* strain and its isogenic Δ*tarO* strain following addition of a small molecule. Compounds that inhibit a conditionally essential enzyme in the WTA pathway were expected to prevent the growth of the WTA-expressing wildtype strain but not of the Δ*tarO* mutant, which cannot initiate polymer synthesis (see Table 1). Using this screen, we have discovered a WTA biosynthesis inhibitor, 7-chloro-*N*,*N*-diethyl-3-(phenylsulfonyl)-[1,2,3]triazolo[1,5-*a*]quinolin-5-amine (1835F03), that prevents the growth of both methicillin-sensitive and methicillin-resistant *S. aureus*. We have identified the target of this molecule as the transmembrane component (TarG) of the ABC transporter that exports WTAs to the cell surface. This is the first small molecule antibiotic that specifically targets WTA biosynthesis.

RESULTS AND DISCUSSION

High-Throughput Screen for WTA Inhibitors Yields a New Antibiotic. A library of 55,000 small molecules was screened in duplicate at a concentration of 38 μM against a wildtype *S. aureus* strain (RN4220) and the

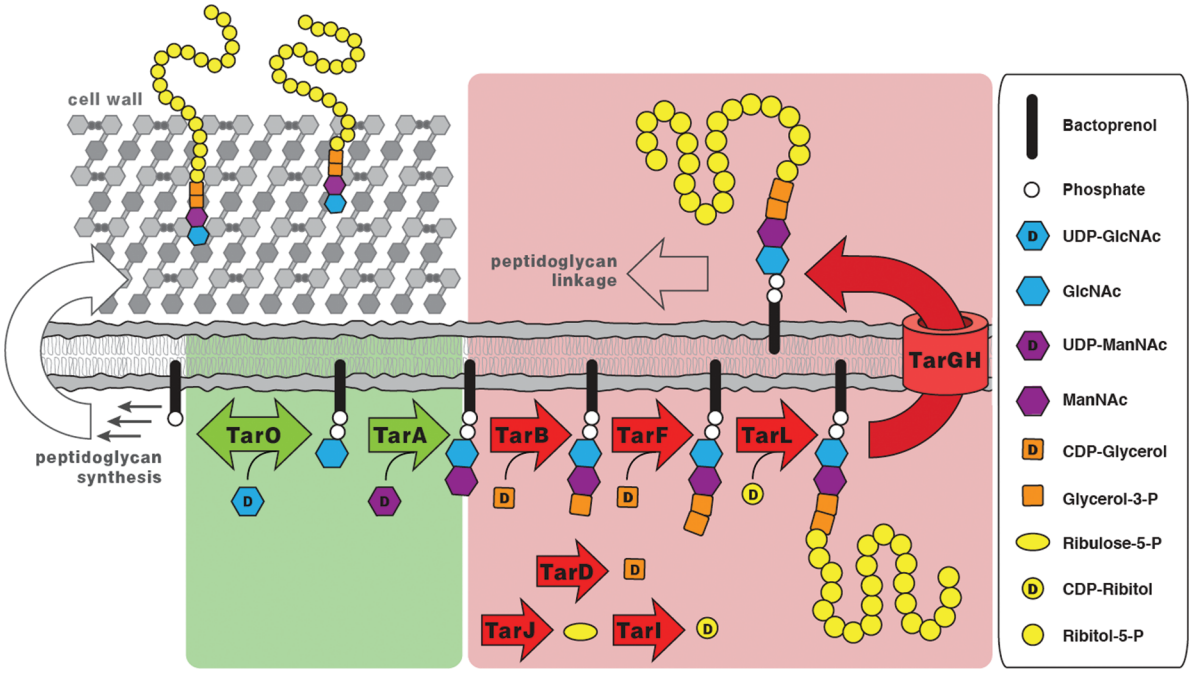

Figure 2. Schematic of the primary *Staphylococcus aureus* WTA biosynthetic pathway (*21, 22*). Following intracellular assembly, the poly(ribitol-phosphate) polymer is transported to the outside by a two-component ABC transporter, TarGH, and then covalently linked through a phosphodiester bond to the MurNAc sugars of peptidoglycan by an unidentified enzyme (*20, 40, 41*). Non-essential WTA pathway enzymes are colored green, and their deletion leads to an avirulent phenotype. Conditionally essential enzymes are colored red, and their deletion is lethal in a wild-type background but permitted in a Δ*tarO* or Δ*tarA* background (*13, 16, 23*). The tailoring enzymes that modify the poly(ribitol-phosphate) polymer are omitted for clarity (*20*). In addition to TarI, J, and L, all *S. aureus* strains contain a homologous set of enzymes (designated TarI′, J′, and K) that directs the synthesis of a distinct WTA polymer; their cellular functions remain incompletely understood.

corresponding Δ*tarO* strain using optical density to monitor growth. We obtained 45 initial hits and confirmed three lead compounds that inhibited the growth of the wildtype strain but had no activity against the Δ*tarO* strain (Table 1). The most potent compound, 1835F03, had a minimum inhibitory concentration (MIC) of 1.3 μg mL^{-1} (3 μM) against the wildtype screening strain. The *in vitro* MIC values against all tested *S. aureus* strains, including five MRSA strains, were also in the low μg mL^{-1} range (Figure 3 and Supplementary Figure 1), but the compound had no activity against other Gram-positive strains that contain WTAs (MIC > 40 μg mL^{-1}), including *Streptococcus pneumoniae*, *Bacillus subtilis* 168, and *B. subtilis* W23. Compound 1835F03 was taken forward for further characterization.

Antibiotic Activity Requires Flux into the WTA Biosynthetic Pathway. We first used the natural product tunicamycin to verify that growth inhibition by

TABLE 1. Conceptual basis for the pathway-specific, high-throughput screen[a]

Small molecule \ Cell type	Wildtype *S. aureus*	Δ*tarO* *S. aureus*
does not inhibit any cellular process	High OD_{600}	High OD_{600}
inhibits an essential cellular process	Low OD_{600}	Low OD_{600}
inhibits a WTA essential gene product	Low OD_{600}	High OD_{600}

[a]Inhibitors of conditionally essential WTA enzymes prevent the growth of the wildtype strain but not the Δ*tarO* strain.

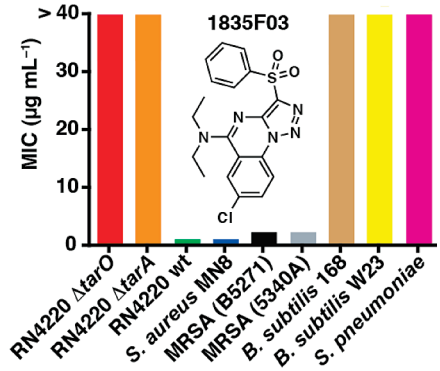

Figure 3. 1835F03 MIC data for a panel of Gram-positive strains, including MRSA clinical isolates B5271 and 5340A. Growth was monitored in triplicate after 12 h at 28 °C using optical density.

1835F03 requires flux into the WTA pathway. Although toxic at high concentrations due to inhibition of MraY, an essential peptidoglycan biosynthetic enzyme (*24*), tunicamycin selectively inhibits TarO at low concentrations (*25*). Therefore, it can be used to block WTA expression at concentrations that have no effect on bacterial growth (Figure 4, panel a and Supplementary Figure 2). We reasoned that if 1835F03 inhibits a conditionally essential WTA pathway enzyme, then tunicamycin should antagonize its antibiotic activity by preventing flux into the biosynthetic pathway. The growth of *S. aureus* RN4220 as well as several clinical isolates was monitored in a two-dimensional matrix of different concentrations of tunicamycin and 1835F03 (*26*). Tunicamycin provided complete protection against 1835F03 in all *S. aureus* strains examined (Figure 4, panel b and Supplementary Figure 3). To confirm the pharmacological results, we constructed a strain of *S. aureus* that expresses *tarO* under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (*22*). In the presence of IPTG-induced WTA biosynthesis, 1835F03 inhibited cell growth; in its absence, 1835F03 had no effect (Figure 4, panel c). These pharmacological and genetic results are consistent in showing that the antibiotic activity of 1835F03 requires flux into the WTA biosynthetic pathway; therefore, they imply that the compound inhibits one of the conditionally essential WTA enzymes.

Identification of the Antibiotic Target. We next tested 1835F03 against conditionally essential *S. aureus* WTA enzymes for which we have previously established *in vitro* biochemical assays, namely, TarBDFI and L (*21*). None of these enzymes was inhibited by 1835F03 at concentrations well above the MIC (100 μM, see Supplementary Figures 4–8). These results narrowed the possible targets of 1835F03 to the two-component ABC transporter that exports WTAs to the bacterial cell surface (TarGH) or to the unidentified ligase that couples exported WTAs to peptidoglycan. Since the transporter has not been reconstituted *in vitro*, we used a genetic approach to query whether 1835F03 inhibits TarGH.

Overexpression of TarGH in *S. aureus* caused a reproducible increase in the MIC (Figure 5), consistent with

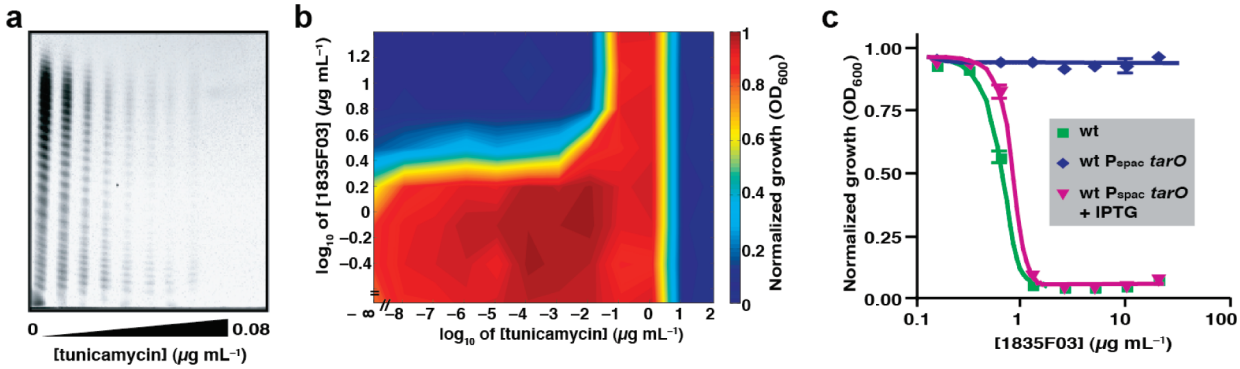

Figure 4. 1835F03 targets the WTA biosynthetic pathway. a) PAGE analysis of WTAs isolated from *S. aureus* RN4220 grown in increasing but sublethal concentrations of a TarO inhibitor, tunicamycin, reveals a dose-dependent decrease in WTA biosynthesis. b) Two-dimensional drug interaction analysis between tunicamycin and 1835F03 in *S. aureus* RN4220 grown for 18 h at 30 °C. At sublethal concentrations that fully inhibit TarO (10^{-2} to 10^{1} μg mL^{-1}), tunicamycin antagonizes the lethal effect of 1835F03. At higher concentrations, tunicamycin is toxic because it inhibits cell wall biosynthesis. c) Growth as a function of 1835F03 concentration for wildtype *S. aureus* RN4220 (■) and the IPTG-inducible *tarO* strain in the absence (◆) and presence (▼) of IPTG shows that antibiotic activity depends on *tarO* expression. Data were normalized to the DMSO controls for each strain.

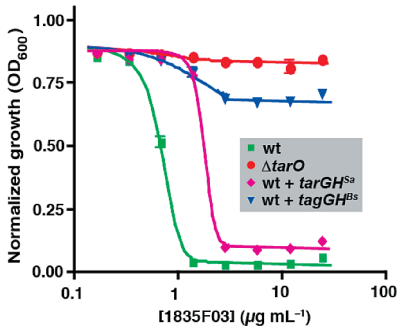

Figure 5. Expression of *B. subtilis* 168 *tagGH*, encoding a two-component WTA transporter, in *S. aureus* RN4220 confers complete resistance to 1835F03. Overexpression of the *S. aureus* WTA transporter (*tarGH*) confers partial resistance, resulting in a 2-fold increase in the MIC of 1835F03. Cells were grown at 28 °C for 12 h and monitored for growth using optical density (OD_{600}). Data were normalized to the DMSO controls for each strain.

this two-component transporter being the molecular target. To confirm this finding, the *B. subtilis* 168 WTA transporter complex TagGH was used for heterologous complementation. *B. subtilis* 168 TagG shares only 58% identity with *S. aureus* TarG, and the transporter has been shown to have promiscuous substrate specificity, exporting poly(glycerol-phosphate) WTAs as well as other structurally distinct polymers (*27*, *28*). We reasoned that if the resistance of *B. subtilis* 168 to 1835F03 is due to an intrinsically resistant ABC transporter (Figure 3, >40 μg mL^{-1}), then the functional expression of this transporter in *S. aureus* should likewise confer resistance. Indeed, expression of *B. subtilis* 168 TagGH in *S. aureus* conferred complete resistance to 1835F03 (Figure 5). These overexpression and heterologous complementation results imply that the two-component transporter TarGH is the molecular target of 1835F03.

To probe whether 1835F03 interacts with the transmembrane component (TarG) or the ATPase component (TarH) of the ABC transporter, we selected resistant mutants on agar plates (at a frequency of 1 in 10^6 cells) and identified a set that remained resistant after propagation in the absence of the compound. Since the first two genes in the pathway are non-essential and removing them confers resistance to 1835F03, it seemed likely that the resistant mutant population would consist of two types: null mutations in *tarO* or *tarA* that prevent initiation of WTA synthesis and mutations in the molecular target of 1835F03. Since *S. aureus* bacteriophage use WTAs as receptors (*29*), we were able to rapidly group the mutants into two classes based on their susceptibility to phage infection (Figure 6, panel a). We selected three phage-resistant and two phage-sensitive mutants for further analysis. As expected, the phage-resistant mutants did not contain extractable WTAs (Figure 6, panel b) (*22*). Targeted sequencing of the *tar* genes from these mutants showed that all three phage-resistant strains contained partial gene deletions or frameshift mutations in either *tarO* or *tarA*. In contrast, the two phage-sensitive mutants contained extractable WTAs. Targeted sequencing of the 1835F03-resistant but phage-sensitive (WTA+) mutants showed that each contained a unique point mutation in *tarG*. The mutant *tarG* alleles encode different amino acid changes (TarG: F82L and TarG:W73C) on the same face of a predicted membrane spanning helix (Supplementary Figure 9). The changes apparently impart resistance to 1835F03 while maintaining functional WTA ABC-transporter activity. To verify that the observed mutations in TarG confer resistance, we exchanged the wildtype allele with each of the mutant *tarG* alleles (*30*). The mutant strains were fully resistant, showing that the point mutations alone are sufficient to bestow 1835F03 resistance in an otherwise wildtype background (Figure 6, panel c) and implicating TarG as the target. In the presence of 1835F03, the TarG mutant strains produced WTAs identical to those produced in the wild-type strain (data not shown). These results confirm that the mutations are within the target rather than simply suppressor mutations that allow for the transport of truncated WTAs (which would occur if a conditionally essential enzyme upstream of TarG were inhibited).

Inhibiting WTA Biosynthesis Arrests Bacterial Growth. We next sought to assess the antibacterial properties of 1835F03. Kill-curve analysis (Figure 7) shows that its activity is fully bacteriostatic: treated *S. aureus* cells immediately cease to divide, but the number of viable bacteria remains constant even at high multiples of the MIC or prolonged exposure (Supplementary Figure 10). Furthermore, if 1835F03 is removed or if tunicamycin is added to block initiation of nascent WTA polymers, bacterial growth resumes after a lag (Figure 7). The prolonged but reversible stasis caused by 1835F03 is consistent with growth arrest, a bacterial response in which cell division and most other metabolic processes cease as the organism seeks to survive

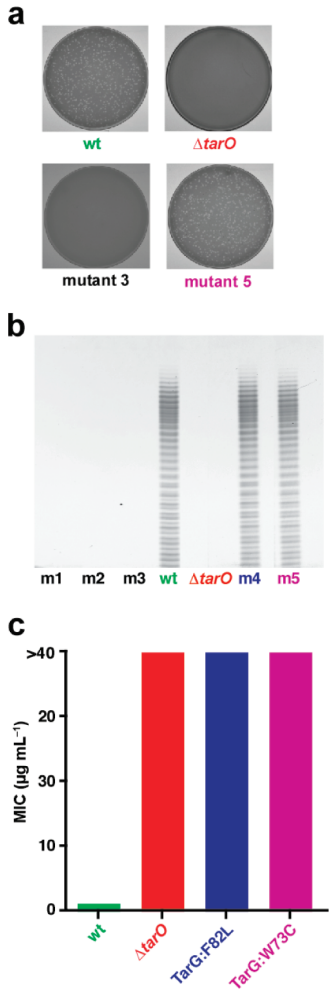

Figure 6. Resistant mutant analysis identified TarG is the target of 1835F03. a) 1835F03-resistant mutants were analyzed for phage-susceptibility to identify WTA producers and WTA non-producers. Mutant 3 is phage-resistant as is the Δ*tarO* strain; mutant 5 is phage-resistant as is the wildtype strain. b) WTA PAGE analysis of the mutants shows that phage infectivity correlates with WTA expression. Strains m1, m2, and m3 were found to have null mutations (m1 has a point mutation in *tarA*, m2 contains a frameshift that led to a premature stop codon in *tarA*, and m3 contains a rearrangement in *tarO* producing a truncated form of the protein). Strains m4 and m5 were found to contain point mutations in *tarG*. c) MIC analysis shows that mutant alleles encoding TarG:F82L (m4) and TarG:W73C (m5) confer complete resistance to 1835F03 in a wildtype background. MICs for the wildtype and Δ*tarO* strains are shown for comparison.

stressful conditions (*31*). Since preventing completion of WTA synthesis once it has initiated is expected to lead

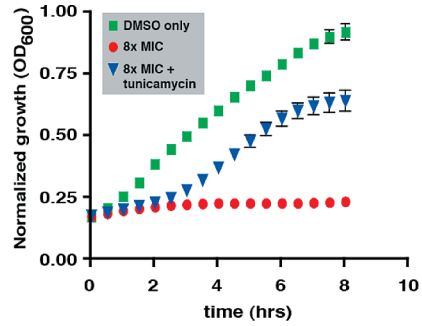

Figure 7. Kill curve analysis of 1835F03 shows a bacteriostatic effect. Compound was added to wells containing *S. aureus* RN4220, and optical density was monitored over time at 30 °C (for colony count data, see Supplementary Figure 10). Tunicamycin, added after 1 h to a 1835F03-treated culture, restores growth.

to an accumulation of bactoprenol-linked WTA precursors, and since bactoprenol levels are limited in bacterial membranes (*32*), blocking WTA synthesis would indirectly lead to a decrease in other bactoprenol-linked metabolites, including peptidoglycan precursors. Decreased pool levels of peptidoglycan precursors may elicit the observed growth stasis response. Consistent with this, growth stasis is also observed upon treatment of *S. aureus* with bacitracin (Supplementary Figure 11), a peptide natural product that sequesters bactoprenol in a trimolecular zinc−bactoprenol−pyrophosphate complex, which prevents its recycling (*33*). Likewise, depletion of mevalonate (a precursor of bactoprenol) biosynthetic genes in *S. aureus* triggers downregulation of primary metabolism transcripts (*34*), while limiting amounts of mevalonate pools in *Streptococcus pneumoniae* results in growth stasis (*35*). It is not known whether bacteria can sense levels of peptidoglycan precursors directly or sense abrupt decreases in rates of peptidoglycan synthesis in comparison with other metabolic processes.

Stasis may alternatively be a result of a programmed stress response to the intracellular accumulation of bactoprenol-linked WTA intermediates. There is evidence that the accumulation of bactoprenol-linked saccharides is deleterious in other organisms (*8–12*). Furthermore, Silhavy and co-workers have shown that the accumulation of bactoprenol-enterobacterial common antigen intermediates in *E. coli* activates two well-characterized, two-component signaling pathways involved in the response to envelope stress (*36*). Other

bacteria may also contain signaling systems that respond to envelope stress caused by inhibition of bactoprenol-dependent metabolic pathways. Consistent with the proposal that inhibiting WTA biosynthesis elicits a stress response, Brown and co-workers have reported that depletion of TarB, D, and F in *Bacillus subtilis* leads to increased transcription from the promoter P_{ywaC}, which drives expression of a putative GTP pyrophosphokinase (*37*). GTP pyrophosphokinases produce a signaling molecule, (p)ppGpp, that is linked to the stringent response (*38*). We are currently using 1835F03 as a chemical probe to elucidate the molecular mechanisms involved in the growth stasis of *S. aureus* in response to WTA inhibition.

Summary and Implications. We have described a general strategy to discover small molecules that inhibit conditionally essential enzymes in non-essential metabolic pathways. The strategy has been applied to WTA biosynthesis but can be adapted to the apparently large number of other pathways that show a similar mixed dispensability pattern. Some of these pathways are potential antibacterial or antivirulence targets, and small molecule inhibitors could therefore have therapeutic utility. More generally, such inhibitors may serve as probes to investigate the cellular effects of blocking particular pathways and may provide insight into signaling pathways involved in sensing and responding to metabolic stresses.

We have demonstrated the utility of our discovery strategy by identifying a small molecule that targets a conditionally essential enzyme involved in WTA biosynthesis in *S. aureus*. The molecular target of the compound is the ABC transporter that exports WTAs from the cytoplasm to the cell surface. We have shown that the molecule, 1835F03, inhibits the growth of *S. aureus*, including MRSA, providing the first pharmacological validation of the WTA pathway as an antibacterial target. Since the pathway is non-essential, the resistance frequency *in vitro* due to null mutations in one of the first two genes is relatively high, but these mutants are not expected to contribute to resistance *in vivo*. Therefore, inhibitors of conditionally essential WTA enzymes may have efficacy *in vivo*, and we are investigating this possibility.

METHODS

Strains and Growth Conditions. Plasmids were constructed in *Escherichia coli* Novablue (Novagen) cells and introduced into the restriction negative *S. aureus* strain RN4220 by electroporation (*39*). Allelic exchange was performed as previously described by Bae *et al.* (*30*) and Meredith *et al.* (*22*). All genetic manipulations were verified either by restriction digest or DNA sequencing. *S. aureus* was grown in tryptic soy broth, and antibiotic markers were selected with erythromycin (Em; 10 μg mL^{-1}), tetracycline (Tc; 2.5 μg mL^{-1}), and chloramphenicol (Cm; single copy integrated into genome 5 μg mL^{-1}, plasmid 10 μg mL^{-1}). Bacterial strains used are listed in Supplementary Table 1. All cloning materials are listed in Supplementary Table 2.

WTA Inhibitor Screen and Hit Follow-Up. Although each strain was marked with a unique fluorescence marker to provide an alternative readout to bacterial growth, optical density proved to be a more robust measure of growth and was used for hit determination. For more thorough details on the fluorescence-based screen, please refer to Supporting Information. On the night before screening, a single colony of each strain was grown in appropriate media. On the day of screening, 384-well plates (Corning 3710) were filled with 40 μL of media. Small molecules (10 mM in DMSO) were transferred from library plates to these culture plates using a 300 nL pin transfer. Following transfer of the small molecules, each plate was inoculated with 40 μL of media containing an equal number of cells. After inoculation, each plate was covered (Corning 3009), stacked five plates high, and incubated at 30 °C for 18 h. For each plate, positive control wells (Em at 10 μg mL^{-1}) and negative control wells (media only) were included in columns 23 and 24.

The following day, the plates were read for OD_{600} using a Perkin-Elmer Envision plate reader. Data were collected and analyzed to identify compounds that killed the wildtype strain but not the Δ*tarO* mutant. Data workup included normalizing the OD data to the positive and negative controls to determine a normalized percent survival for each plate. A hit was defined as greater than 50% survival for the Δ*tarO* mutant and less than 10% survival for the wildtype strain based on the OD data.

For a secondary screen, hits from the primary screen were evaluated against wildtype and the Δ*tarO* mutant as described above, but the compound was investigated using a four point dose−response curve starting at concentrations of 50 μM with 2-fold serial dilutions. Three inhibitors were validated. One such small molecule was 1835F03 (see Supporting Information for compound characterization data).

Production of 1835F03 Mutants. 1835F03-resistant mutants were selected both in liquid culture and on solid media. Mutants were selected on solid media by plating ~5×10^6 CFUs on TSB agar plates containing 1835F03 at 4× MIC. Colonies that were fully grown after 48 h were passaged four times in the absence of 1835F03 and evaluated for 1835F03 sensitivity, and stable mutants were analyzed for the production of WTAs using phage infection and WTA extraction as previously described (*22*).

Mutants prepared in liquid culture were first colony-purified on TSB agar plates. Individual colonies were grown in culture and passaged four times in the absence of 1835F03. The culture was then colony purified, evaluated for 1835F03 sensitivity, and analyzed for the production of WTAs using phage infection and WTA extraction as previously described (*22*).

***In Vitro* Enzyme Inhibition Assays.** *S. aureus* TarB, D, F, I, and L were cloned, overexpressed, and purified as described previ-

ously (*21*). Radiometric *in vitro* assays were carried out as previously described (*21*) to test the inhibitory effects of 1835F03 on Tar B, D, F, and L. See Supporting Information for exact reaction conditions. Briefly, enzymes were tested at 200 nM, and 1835F03 was added to the reactions at 5, 50, or 100 μM. In each case, a reaction with no 1835F03 served as a negative control (active enzymatic reaction) and a reaction with heat-treated enzyme served as a positive control (no enzymatic reaction). 1835F03 was incubated with all reaction components except the substrates at RT. After 10 min, substrates were added, and the reactions were allowed to proceed for 60 min. The reactions were quenched with DMF, and the TarB, D, F, and L products were imaged as previously described (*21*). TarI reactions were monitored by HPLC. See Supplementary Figures 4−8 for results.

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

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