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ARTICLE

# Synthetic Lethal Compound Combinations Reveal a Fundamental Connection between Wall Teichoic Acid and Peptidoglycan Biosyntheses in *Staphylococcus aureus*

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**ABSTRACT** Methicillin resistance in *Staphylococcus aureus* depends on the production of *mecA*, which encodes penicillin-binding protein 2A (PBP2A), an acquired peptidoglycan transpeptidase (TP) with reduced susceptibility to β-lactam antibiotics. PBP2A cross-links nascent peptidoglycan when the native TPs are inhibited by β-lactams. Although *mecA* expression is essential for β-lactam resistance, it is not sufficient. Here we show that blocking the expression of wall teichoic acids (WTAs) by inhibiting the first enzyme in the pathway, TarO, sensitizes methicillin-resistant *S. aureus* (MRSA) strains to β-lactams even though the β-lactam-resistant transpeptidase, PBP2A, is still expressed. The dramatic synergy between TarO inhibitors and β-lactams is noteworthy not simply because strategies to overcome MRSA are desperately needed but because *neither* TarO *nor* the activities of the native TPs are essential in MRSA strains. The "synthetic lethality" of inhibiting TarO and the native TPs suggests a functional connection between ongoing WTA expression and peptidoglycan assembly in *S. aureus*. Indeed, transmission electron microscopy shows that *S. aureus* cells blocked in WTA synthesis have extensive defects in septation and cell separation, indicating dysregulated cell wall assembly and degradation. Our studies imply that WTAs play a fundamental role in *S. aureus* cell division and raise the possibility that synthetic lethal compound combinations may have therapeutic utility for overcoming antibiotic-resistant bacterial infections.

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Most bacteria are surrounded by a tough polysaccharide matrix known as peptidoglycan (PG). This matrix, which is essential for survival, protects bacteria from lysis due to high internal osmotic pressures. PG biosynthesis is the target of the β-lactams (*e.g.*, penicillin), which are among the safest and most effective antibiotics ever developed for clinical use (*1*). The β-lactams covalently inactivate the transpeptidase (TP) domains of high molecular weight penicillin binding proteins (HMW-PBPs), which cross-link the polysaccharide chains of PG. Unfortunately, resistance to β-lactams is now widespread and has become a particular problem in *Staphylococcus aureus*. Invasive β-lactam-resistant *S. aureus* (methicillin-resistant SA, or MRSA) infections are directly responsible for 20,000 deaths annually in the United States (*2*). Although two new classes of antibiotics have been introduced since 2000 to treat these infections, clinical resistance to both has already been observed (*3, 4*), highlighting the ongoing need for new strategies to overcome MRSA.

The most common mechanism of bacterial resistance to the β-lactams involves inactivation by β-lactamases, and a successful strategy to overcome this form of inactivation by combining a β-lactam and a β-lactamase inhibitor has been used clinically (*5*). Unfortunately, MRSA strains develop resistance through a different mechanism: the acquisition of a β-lactam-resistant TP, PBP2A (*6, 7*). This gene is not native to *S. aureus* (*6*) but was acquired by lateral transfer from another organism (*8*) and has spread widely. When other *S. aureus* PBPs are inhibited by β-lactams, PBP2A compensates by cross-linking the PG polysaccharides that

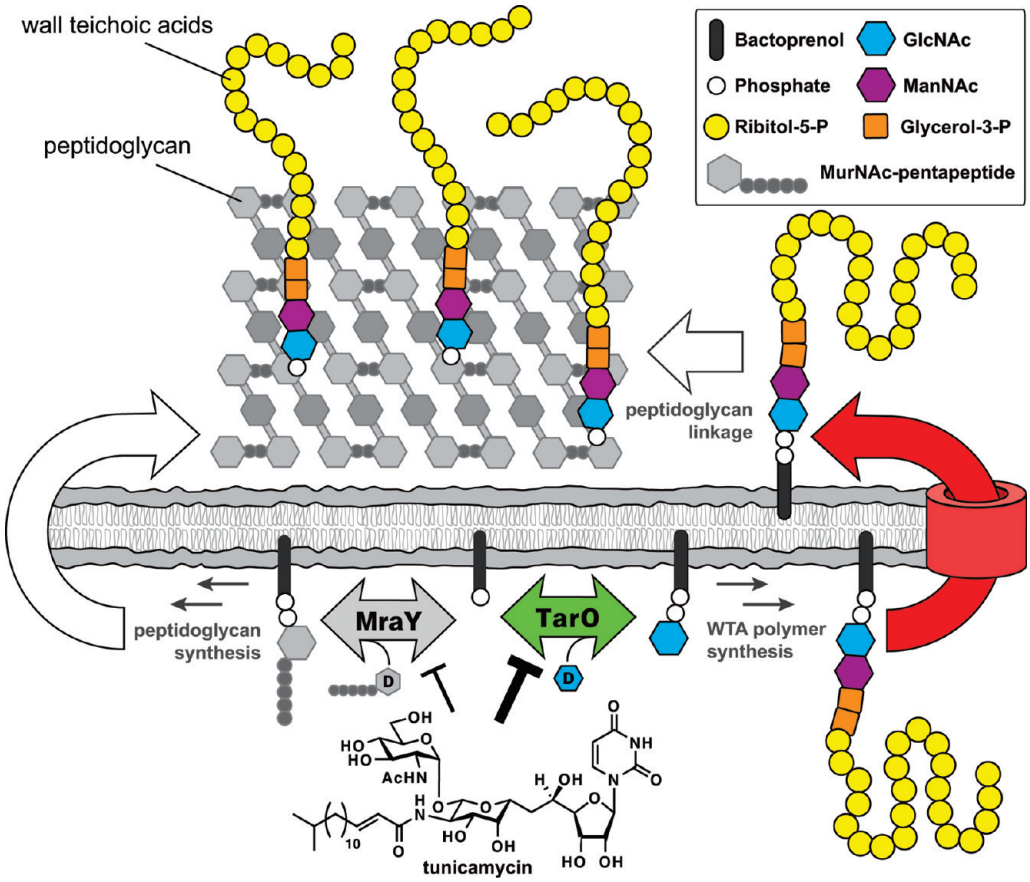


**Figure 1. Both wall teichoic acid (WTA) and peptidoglycan syntheses begin with similar reactions. TarO catalyzes the first step in the WTA biosynthetic pathway and utilizes bactoprenol-phosphate as a substrate; MraY, a related enzyme, catalyzes an essential step in peptidoglycan biosynthesis using the same carrier lipid (*46*). The chemical structure of the natural product inhibitor tunicamycin is shown; D = UDP-activated substrate.**

are produced (*9*). One strategy being explored to overcome β-lactam resistance in MRSA is to develop β-lactam analogues that are capable of inhibiting PBP2A (*10*). An alternative strategy, examined here, is to use existing β-lactams in combination with compounds that inhibit other targets involved in the expression of methicillin resistance in MRSA (*11*).

In 1994, Maki *et al.* used transposon insertional inactivation to identify a gene, *llm*, which seemed to play an important role in methicillin resistance in MRSA (*12*). This gene was reported to encode a multipass transmembrane protein of unknown function. Sequence comparisons suggest that *llm* encodes TarO (also known as TagO), which catalyzes the first step in wall teichoic acid (WTA) biosynthesis in *S. aureus* (*13*). TarO facilitates the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to an undecaprenyl carrier to produce a lipid-linked monosaccharide that is further elaborated into a long anionic polymer comprising ribitol phosphate repeats (Figure 1) (*14–17*). The polymer is subsequently exported from the cytoplasm and coupled to PG, resulting in a cell envelope containing layers of PG densely functionalized with negatively charged WTAs (*18*). WTAs are not essential for *S. aureus* survival *in vitro* since *tarO* can be deleted, however, they are critical for establishing infections in some animal models (*19, 20*), and it has been suggested that WTAs are virulence factors required for *Staphylococcal* adhesion to host tissue (*21*).

Here we use both genetic and pharmacological approaches to show that blocking TarO, and thus preventing WTA expression, specifically sensitizes MRSA strains to β-lactams. The β-lactam susceptibility is due to the

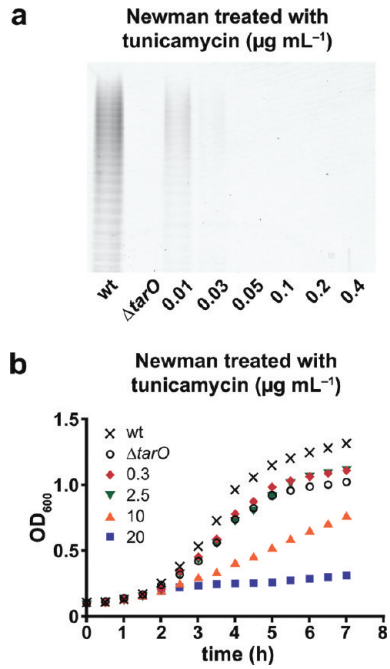


**Figure 2. Tunicamycin selectively inhibits TarO in *S. aureus*. a) Representative gel showing levels of WTAs extracted from *S. aureus* strain Newman treated overnight with varying concentrations of tunicamycin. WTA expression is abolished at 0.1 μg mL$^{-1}$; wt = Newman wildtype in the absence of tunicamycin. b) Growth curves of *S. aureus* strain Newman in the presence of various concentrations of tunicamycin. High concentrations of tunicamycin inhibit MraY, leading to growth inhibition. However, strains treated with lower concentrations of tunicamycin that abolish WTA expression grow similarly to the Δ*tarO* mutant.**

combined inactivation of the native PBPs and TarO, two classes of targets that have nonessential enzymatic activities in MRSA. The synthetic lethality of this compound combination suggests that ongoing WTA expression is coupled to the assembly of PG, and we present evidence that supports this hypothesis. The work described here provides a possible new strategy for treating MRSA infections by combining a β-lactam and a TarO inhibitor, and also reveals that ongoing WTA expression is required for properly coordinated cell division in *S. aureus*.

## RESULTS AND DISCUSSION

**WTA Expression Is Required for Methicillin Resistance in MRSA.** Transposon insertion in a gene of unknown function, *llm*, was shown to reduce β-lactam resistance and increase Triton X-induced autolysis in a wide variety of MRSA strains (*12*). The reported gene sequence is identical to the gene sequence for *S. aureus tarO*, suggesting that WTA expression is required for high-level methicillin resistance. To confirm that WTAs do indeed play a role in methicillin resistance, we generated a *tarO*-null mutant of MW2 (see SI), a well-characterized MRSA strain that displays moderate resistance to β-lactams. This mutant strain shows increased Triton X-induced autolysis (not shown) and is approximately 8-fold more sensitive than the parent strain to β-lactam antibiotics, including methicillin, imipenem, ceftazidime, and cephradine (Supplementary Table 2). These results confirm that the expression of WTAs is required for methicillin resistance in MRSA *in vitro*. Preventing WTA biosynthesis also restored susceptibility to β-lactams in an *in vivo C. elegans* MRSA infection model (Supplementary Figure 1). These studies imply that small molecules that block WTA expression should sensitize MRSA strains to β-lactams.

**Tunicamycin Is a Selective Inhibitor of *S. aureus* WTA Expression.** Tunicamycin is a natural product inhibitor of a large family of enzymes that transfer hexose-1-phosphates from nucleotide-sugar donors to membrane-embedded lipid phosphates to form lipid-pp-hexose products (Figure 1) (*22*). In *S. aureus*, this family of enzymes includes both TarO and MraY, an essential enzyme involved in PG biosynthesis. Tunicamycin is known to be selective for transferases that use UDP-GlcNAc as a substrate over transferases that use other UDP-sugars as substrates (*22*). To characterize the selectivity of tunicamycin for inhibition of TarO over MraY, we grew several *S. aureus* strains, including both methicillin-sensitive (MSSA) and MRSA strains, in increasing concentrations of tunicamycin and then analyzed extracted WTAs using silver-stained PAGE (*23*). WTA expression began to decrease at tunicamycin concentrations of 0.02 μg mL$^{-1}$ and WTAs were undetectable in all strains at concentrations of 0.2 μg mL$^{-1}$ (Figure 2, panel a and Supplementary Figure 2).

Exponential phase growth rates were measured for the same set of *S. aureus* strains in the presence of increasing concentrations of tunicamycin (Figure 2, panel b and Supplementary Figure 3). Growth rates for available Δ*tarO* strains were also measured and showed modest defects compared with the wildtype strains. The tunicamycin-treated strains grew identically to the Δ*tarO* controls even at concentrations 16-fold greater than those required to abolish WTA biosynthesis. At higher concentrations, growth inhibition was observed as tuni-

camycin began to inhibit MraY (MICs ranged from 10 to 40 μg mL$^{-1}$). Thus, there is a large window between the tunicamycin concentrations required to fully inhibit TarO and those that hamper growth through MraY inhibition. Consistent with this, WTA-inhibitory concentrations of tunicamycin that do not affect growth rates (0.4 μg mL$^{-1}$) had no effect on [$^{14}$C]-GlcNAc incorporation into *S. aureus* PG but dramatically reduced labeling of the extracted WTA fraction compared with the untreated controls (Supplementary Table 3). These experiments fully validate tunicamycin as a selective chemical probe to study the effects of inhibiting WTA expression in *S. aureus*.

**Tunicamycin Inhibition of WTA Expression Sensitizes MRSA Strains to β-Lactams.** To assess whether pharmacological inhibition of WTA expression sensitizes MRSA to β-lactams, we tested the β-lactam MICs of several MRSA strains in the presence of tunicamycin in a checkerboard assay. The heat map for a representative MRSA strain treated with tunicamycin and the β-lactam oxacillin shows biphasic behavior in response to the presence of tunicamycin (Figure 3, panel a). That is, the oxacillin MIC for the strain, clinical isolate MRSA$^+$, decreases abruptly from 25 to 0.4 μg mL$^{-1}$ at a tunicamycin concentration of 0.08 μg mL$^{-1}$ and then plateaus over a wide concentration range before dropping again at tunicamycin concentrations exceeding 10 μg mL$^{-1}$. This biphasic behavior is consistent with the selective tunicamycin inhibition of a nonlethal target followed by the subsequent inhibition of a lethal target. Similar behavior is observed for other MRSA strains and β-lactams (Supplementary Figure 4). We infer that the dramatic β-lactam sensitization (synergy) observed at low tunicamycin concentrations is due to TarO inhibition, whereas the lethal effects of tunicamycin at high concentrations are related to inhibition of MraY.

We also measured the MICs of several MRSA strains against a panel of β-lactams in the presence or absence of tunicamycin at a concentration that inhibits WTA expression but not PG synthesis (0.4 μg mL$^{-1}$). The tunicamycin-treated strains showed increased sensitivity to all β-lactams tested (Table 1). Kill curve analysis in MRSA strain COL showed that when WTA synthesis is prevented, methicillin becomes bactericidal at concentrations that normally do not affect growth (Figure 3, panel b). Transcriptomes of COL treated with tunicamycin and methicillin alone or in combination were also consistent with the data showing that tunicamycin sensitizes MRSA strains to β-lactams (Supplementary Tables 5 and 6). That is, the compound combination, but not the individual compounds, induced a massive cell wall stress response that is similar to those observed for susceptible *S. aureus* strains treated with β-lactams only (*24–28*).

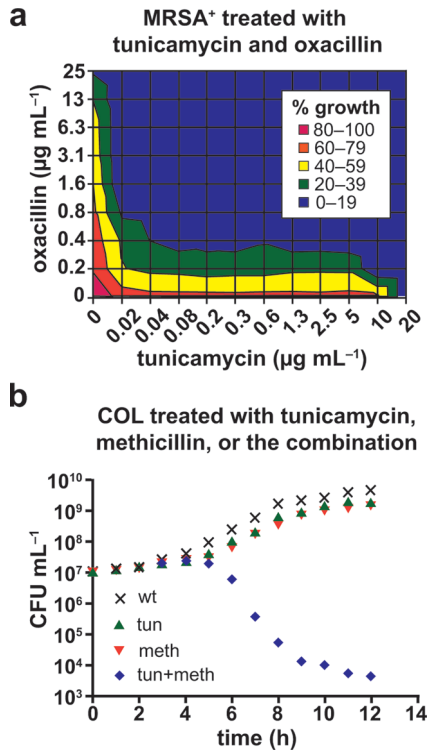


**Figure 3. Methicillin and tunicamycin comprise a synthetic lethal combination in methicillin-resistant *S. aureus*. a) Checkerboard drug-interaction analysis in MRSA clinical isolate MRSA$^+$ shows that tunicamycin, at concentrations that abolish WTA synthesis without affecting growth, sensitizes MRSA to the β-lactam oxacillin. b) Kill curve analysis of methicillin (400 μg mL$^{-1}$) and tunicamycin (0.4 μg mL$^{-1}$) in MRSA strain COL shows a bactericidal mechanism of killing; methicillin was added to the tunicamycin-treated culture after 3 h.**

**TarO Inhibition Does Not Sensitize MRSA Strains to Other Classes of Antibiotics.** To determine whether tunicamycin-treatment renders MRSA strains susceptible to other classes of antibiotics, we measured MICs for several, including chloramphenicol, which inhibits protein synthesis, ciprofloxacin, which inhibits DNA synthesis, and vancomycin, cycloserine, and moenomycin, all of which inhibit PG biosynthesis by mechanisms different

**TABLE 1. MRSA strains are sensitized to β-lactams when WTA biosynthesis is inhibited by tunicamycin at 0.4 μg mL$^{-1}$**

| | MW2 MIC (μg mL$^{-1}$) | | | MRSA$^{+}$ MIC (μg mL$^{-1}$) | | | 1784A MIC (μg mL$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| tunicamycin | − | + | fold Δ | − | + | fold Δ | − | + | fold Δ |
| cefotaxime | 12.5 | 1.6 | 8 | 25 | <0.8 | >32 | 12.5 | 1.6 | 8 |
| cefoxitin | 25 | 6.3 | 4 | 50 | 6.3 | 8 | 50 | 12.5 | 4 |
| ceftazidime | 100 | 12.5 | 8 | >100 | 6.3 | >16 | 100 | 6.3 | 16 |
| cephradine | 100 | 6.3 | 16 | 200 | 6.3 | 32 | 100 | 3.1 | 32 |
| imipenem | 0.4 | 0.1 | 4 | 1.6 | <0.1 | >16 | 1.6 | <0.1 | >16 |
| methicillin | 12.5 | 3.1 | 4 | 100 | <3.1 | >32 | 50 | 3.1 | 16 |
| oxacillin | 25 | 0.4 | 64 | 25 | 0.4 | 64 | 50 | 0.4 | 128 |
| cycloserine | 50 | 25 | 2 | 100 | 50 | 2 | 100 | 50 | 2 |
| moenomycin | 0.03 | 0.03 | 0 | 0.03 | 0.03 | 0 | 0.03 | 0.03 | 0 |
| vancomycin | 3.1 | 3.1 | 0 | 3.1 | 3.1 | 0 | 3.1 | 3.1 | 0 |

from those of the β-lactams (Table 1 and Supplementary Table 4). At concentrations that inhibit WTA expression but do not affect PG biosynthesis, tunicamycin had no effect on the MICs of these antibiotics against MRSA. Thus, TarO inhibition does not increase cell envelope permeability to antibiotics that have intracellular targets nor does it generally enhance MRSA susceptibility to antibiotics that have cell wall targets. The sensitizing effects of TarO inhibition in MRSA strains are specific to β-lactams.

**Simultaneous Inhibition of TarO and the Native Transpeptidases Is Synthetically Lethal.** Our results show that tunicamycin and the β-lactams display dramatic synergy against MRSA strains. This synergy is distinct from "antibiotic synergy" (*29–31*) because the latter phenomenon involves two antibiotics that function by inhibiting essential targets. In MRSA strains, the cross-linking functions of the native PBPs are nonessential in the presence of β-lactams since PBP2A performs cross-linking when they are inhibited. Furthermore, TarO is a nonessential target in all *S. aureus* strains, and no MIC for its inhibition can be defined. Although tunicamycin has a secondary, lethal target in MraY, the concentrations that confer β-lactam sensitization are 2 orders of magnitude *below* the concentrations required to inhibit MraY. Furthermore, the concentrations of β-lactams that kill MRSA strains in the presence of tunicamycin are far below those required to inhibit PBP2A. Thus, the synergy observed is not "antibiotic synergy" but "synthetic lethality". Synthetic lethality results when inactivating mutations (or pharmacological inhibition) in two or more genes (or enzymes) has a lethal phenotype even though full inactivation of the individual genes/enzymes is tolerated. This phenomenon implies a functional connection between the inactivated genes/enzymes. Therefore, the synergy between TarO inhibition and inhibition of the native TPs in MRSA strains suggests that WTA biosynthesis is functionally coupled to PG biosynthesis in *S. aureus*.

**Genetic Deletion of the WTA Biosynthetic Pathway Results in Cell Division Defects.** We examined the cellular architectures of several MSSA and MRSA strains and their Δ*tarO* counterparts by transmission electron microscopy (TEM). Figure 4 shows representative electron micrographs of wildtype (panel a) *S. aureus* RN6390 and its Δ*tarO* mutant (panel b; see also Supplementary Figures 5 and 6). The wildtype strains have the characteristic appearance expected for *S. aureus* based on extensive EM studies by others. Cells have a continuous electron-dense (dark-staining) layer at the surface that continues down the center of the division plane as septation progresses. This material has been identified previously as WTA-rich PG (*32*). Although the electron-dense layer at the midzone appears as a single layer in TEM images, cryo-EM studies have shown that it comprises two layers separated by a less dense midzone (*32*). Efficient separation of daughter cells occurs once septation is complete and is proposed to involve the ac-

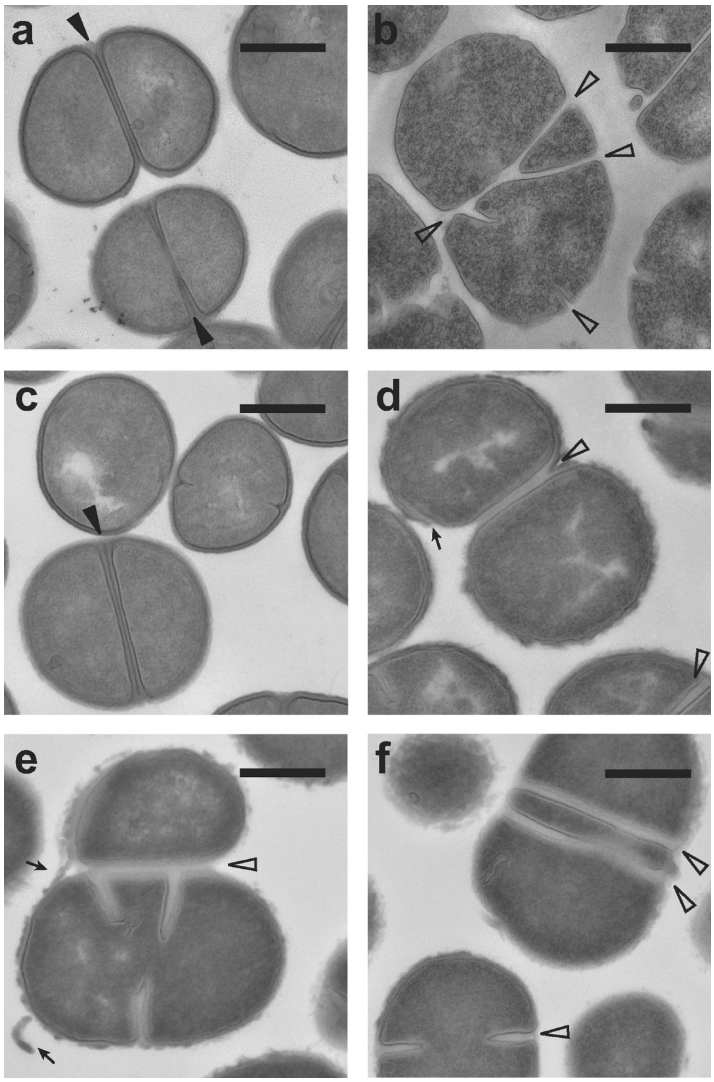


**Figure 4. Electron micrographs of *S. aureus* strains show the effects of the lack of wall teichoic acid (WTA) expression on cell division. a) Wildtype MSSA RN6390 and b) its Δ*tarO* mutant. Cell division in WTA-null strains frequently occurs asymmetrically, and many cells contain duplicate septa. These septa can be parallel or occur at irregular angles, and many are incomplete. In addition, fields of cells contain numerous pseudomulticellular clusters with non-orthogonal division planes. c–f) A time course of electron micrographs of MSSA RN6390 treated with tunicamycin (0.4 μg mL$^{-1}$) reveals that halting WTA expression is detrimental to cell division, leading to misplaced septa and cell separation defects: c) untreated control; d) 1 h; e–f) 2 h (see Supplementary Figures 7 and 8 for time course images of MRSA strain COL treated with tunicamycin). Filled arrowheads point to the splitting system; empty arrowheads point to septa containing low density material; arrows point to secondary cell wall material that is peeling away from the cell surface; scale bars = 500 nm.**

tion of hydrolytic enzymes that degrade cell wall material between the WTA-rich PG layers at the midzone to produce individual cells that each have a continuous WTA-rich surface layer. Separated hemispherical daughter cells rapidly "round up" to produce the characteristic coccoid shape of *S. aureus*, and each subsequent division event occurs along an axis orthogonal to the recently completed division plane.

In contrast to their wildtype counterparts, the Δ*tarO* mutants do not have a dark staining surface layer and their edges appear indistinct; furthermore, the material between daughter cells that have completed septation but have not undergone separation is uniformly light and typically thicker than in wildtype cells (Figure 4, panels a and b; see also Supplementary Figures 5 and 6). More strikingly, septation in the Δ*tarO* mutants is defective with respect to placement of new septa and exclusion of additional septa. For example, new division planes frequently initiate at angles that are not orthogonal to the previous division plane, and multiple septa are often observed. These observations indicate that the biosynthetic machinery involved in cell division has become dysregulated. In addition, Δ*tarO* mutants fail to separate efficiently once division is complete, resulting in multicellular clusters. The effects described are seen in both MSSA and MRSA strains, indicating that WTA expression is required for normal cell division in *S. aureus*.

**Time-Dependent Cell Division Defects Occur upon Pharmacological Inhibition of WTA Biosynthesis.** Validated small molecule inhibitors of specific targets not only provide confirmation of ge-

netic results but also can have significant advantages. One particular advantage of using tunicamycin in imaging studies of *S. aureus* is that TarO inhibition is rapid, which means it is possible to follow phenotypic changes as they occur. We examined TEM images of several MSSA and MRSA strains after 0–4 h of growth in the presence of tunicamycin at 0.4 $\mu$g mL$^{-1}$ (Figure 4, panels c–f and Supplementary Figures 7 and 8). After 1 h, most cells are undergoing or have completed a single cell division. Those undergoing division appear relatively normal in that, like wildtype cells, they divide in the middle (compare Figure 4, panels c and d). However, the densely stained material that runs along the midline of the division plane in wildtype cells is completely absent in these tunicamycin-treated cells, supporting the previous identification of this midzone material as WTA-rich PG. In addition, the densely staining surface layer surrounding the older portions of each daughter cell begins to peel away in large sections starting from the site of septal initiation.

At later time points, representing subsequent cell divisions, abnormalities in septal placement are observed, with new division sites initiating along nonorthogonal planes. Partial or complete duplicate septa are frequently observed in the same cell, sometimes right next to each other, but often at angles that cut off a small portion of the cell (Figure 4, panels e and f). The stripping of the electron dense layer from the cell surface, which begins during the first division cycle, continues with each subsequent cycle until the surface layer is completely absent. The terminal phenotypes of the tunicamycin-treated cells closely resemble those seen in the Δ*tarO* mutants, with multiple septal defects and significant numbers of pseudomulticellular clusters in which cells have failed to separate effectively following division (Supplementary Figure 9).

**Ongoing WTA Synthesis Is Required for Proper Septation.** The imaging studies reported here show that ongoing WTA synthesis is required for proper septation, a result consistent with the synthetic lethality of tunicamycin and β-lactam combinations. During normal cell division, *S. aureus* divides along orthogonal planes. It is not known exactly how these orthogonal division planes are established, but it was recently reported that thick rings, or ribs, of PG form prior to centripetal assembly of the septal disks and are retained after division is complete. These ribs are proposed to act as ultrastructural elements that mark the location of previous division planes and thus determine the placement of future division planes (*33*), perhaps by scaffolding proteins involved in cell wall assembly. Like PG elsewhere in the cell, the PG in these ultrastructural elements is likely functionalized with WTAs. Since preventing WTA synthesis leads to misplaced and duplicated septa, we suggest that WTAs also play a scaffolding role in encoding septal placement. Whether the PG ribs form correctly when WTA synthesis is blocked can now be addressed using tunicamycin as a chemical probe.

**Ongoing WTA Synthesis Is Required for Efficient Cell Separation.** Our TEM studies also indicate that ongoing WTA synthesis is required for proper regulation of the "splitting system" involved in cell separation. Cell separation normally occurs through the action of a hydrolytic enzyme complex, which degrades the less dense material between WTA-rich layers once the division plane is complete (*32, 34, 35*). There is evidence that WTAs mask, *via* steric and/or electrostatic mechanisms, enzyme binding and cleavage sites on the PG to which they are attached, thus protecting PG from exogenous and endogenous hydrolases (*36, 37*). It has been suggested that by excluding autolytic enzymes from certain areas, WTAs may regulate PG degradation (*37*). In wildtype *S. aureus,* the WTA-rich layers along the division plane may protect the underlying PG layers from degradation as the autolysins track between them to enact cell separation. In cells lacking WTAs, the hydrolytic machinery does not function at the division site once the septum is complete, and so multicellular clusters with thick septal PG form (Figure 5). Cell separation eventually occurs but is presumed to involve a mechanical process (*34*).

Remarkably, although septal hydrolysis is inefficient when WTAs are absent, the WTA-rich surface layers are stripped off so rapidly in tunicamycin-treated cells that the process must involve some hydrolytic machine (*i.e.*, "stripping system") (*34*). The stripping process appears to occur between the WTA-rich surface layer and an electron-dense membrane layer that is proposed to comprise PG threaded with membrane-bound lipoteichoic acids (LTAs). These LTAs may protect the underlying layers of PG from catastrophic hydrolysis in much the same manner that the WTA-rich layers are proposed to protect septal PG from hydrolysis as the autolysins track between them (Figure 5).

**Conclusion.** It has been appreciated for decades that WTAs are important in the physiology of Gram-

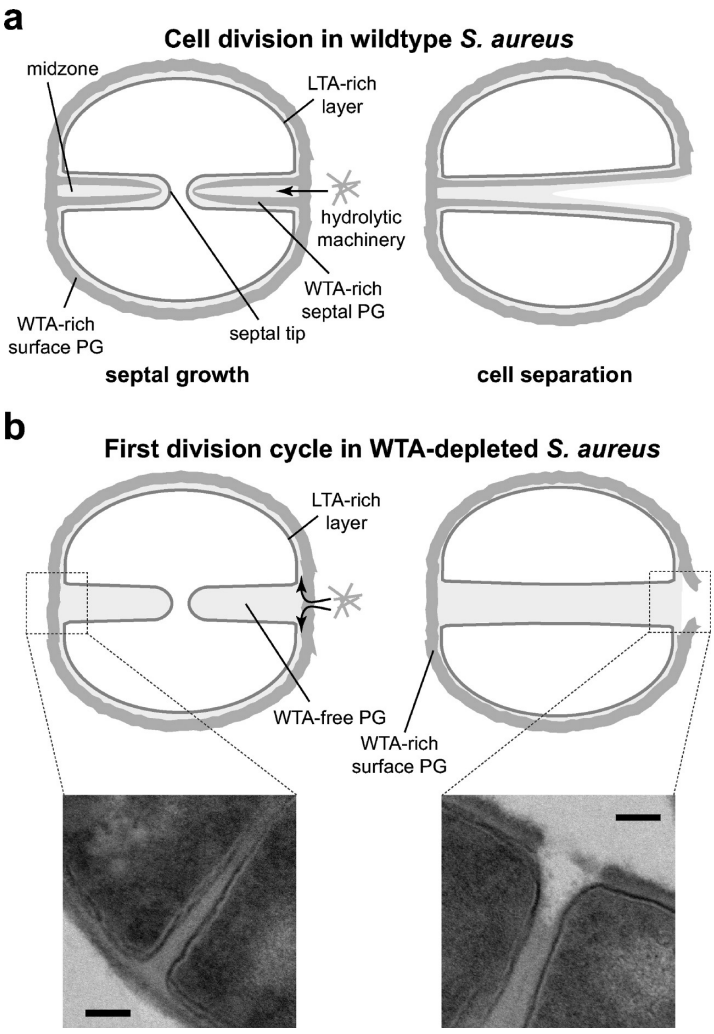


**Figure 5. Models of *S. aureus* cell division. a) Wildtype *S. aureus* cell division model adapted from Beveridge and co-workers (*32*). b) The effect of tunicamycin treatment on septal formation during the first division cycle; the dark staining WTA-rich layer does not extend into the newly formed septum; autolysins are dysregulated by the absence of WTAs and cell separation is hindered, resulting in pseudomulticellular clusters; scale bars =100 nm.**

positive organisms, but specific roles have been difficult to identify because WTAs are highly abundant anionic polymers that affect many cell envelope properties (*13, 17, 18*). Furthermore, these and other functions partially overlap with those of LTAs, a structurally related class of anionic polymers that are anchored in the cell membranes (rather than to the cell walls) of Gram-positive organisms (*17*). Nevertheless, recent studies in the rod-shaped organism *Bacillus subtilis* have suggested that WTAs play an integral role in PG synthesis. For example, deletion of *B. subtilis tagO* leads to the rounding of cells, showing that WTAs are required for proper cell morphology, which depends on correct assembly of the bacterial sacculus (*38*). Moreover, the *B. subtilis* WTA biosynthetic enzymes localize in a helical pattern reminiscent of the organization of nascent PG, and they have been shown to interact with MreC and MreD, which help scaffold PG biosynthetic enzymes. It has thus been suggested that WTA biosynthesis in *B. subtilis* is carried out by a multienzyme complex that is associated with the PG biosynthetic machinery (*39*).

Similar localization studies have not been carried out in *S. aureus*. However, the results reported here, which combine the use of specific small molecule probes for PG and WTA biosynthesis with transmission electron microscopy, show that WTA expression plays a fundamentally important role in cell division in *S. aureus*. We do not yet understand the molecular mechanisms that link WTA and PG synthesis, nor can we yet explain the molecular basis for the synthetic lethality of tunicamycin and a β-lactam. However, we do not think that the bacteria die primarily from a weakened and more accessible cell wall for the following reasons (*40–42*). First, although WTA-null strains do show increased rates of Triton-X-induced autolysis, perhaps because WTA depletion allows activated hydrolases better access to susceptible PG bonds, there is no evidence that such strains spontaneously lyse at significantly increased rates compared with wildtype cells. Second, if β-lactam sensitization merely reflected increased hydrolysis of a weakened cell envelope, one might expect to observe synergy between tunicamycin and

other cell wall active antibiotics that weaken the cell wall, such as vancomycin. At tunicamycin concentrations that affect only WTA synthesis, this is not the case. A final argument against increased hydrolytic susceptibility as the major explanation for the observed tunicamycin-β-lactam synergy is that it does not take into account the dramatic septal defects that occur in both MSSA and MRSA strains upon blocking WTA synthesis. These defects lead us to suggest that the β-lactam sensitization observed in MRSA strains upon WTA inhibition is related to defective assembly of the septal PG biosynthetic machinery.

One possible mechanism for how the defective assembly of PG machinery due to TarO inhibition could result in β-lactam-mediated cell death would involve mislocalization of either PBP2 or PBP2A. Both enzymes must be co-localized in order for β-lactam resistance to be expressed in MRSA. If preventing WTA synthesis affects localization of these PBPs, then β-lactam treatment could become lethal even if PBP2A is expressed (as we know it is) and potentially active (*43*). Indeed, there is precedence for such a mechanism since (−)-epicatechin gallate, a component of green tea that is proposed to affect membrane fluidity, causes mislocalization of PBP2 and decreases β-lactam resistance in MRSA strains (*44*). Tunicamycin, here validated as a highly selective TarO inhibitor, should provide a useful tool for efforts to identify the molecular mechanisms that link WTA and PG biosynthesis in *S. aureus*.

In closing, we emphasize that our studies also have significant implications for the use of synthetic lethal compound combinations to overcome bacterial infections. "Chemical synthetic lethality" is an emerging strategy in cancer chemotherapy (*45*) but has not yet been deliberately exploited to overcome bacterial infections. We have demonstrated here that TarO inhibitors and β-lactams comprise a useful synthetic lethal pair for killing MRSA strains. Although tunicamycin itself has eukaryotic toxicity, it should be possible to identify compounds that better discriminate between bacterial TarO and its eukaryotic orthologs.

## METHODS

**MIC Measurement Protocol.** Minimum inhibitory concentration (MIC) assays were performed as follows. Compound dilutions (1:1) were made in a 96-well PCR plate using sterilized water as solvent and then aliquotted (1.5 μL) into 96-well plates (Corning 3595). An overnight culture of *S. aureus* was diluted 1/1000 into fresh TSB to give *ca.* $5 \times 10^5$ CFU $mL^{-1}$, and 150 μL aliquots were added to each well of the 96-well plate. Erythromycin (10 μg $mL^{-1}$) was used as a positive control (full inhibition of growth), and solvent was used for the negative control (full growth). Plates were shaken at 30 °C for 16–20 h. The optical densities of the wells were measured on a plate reader and normalized to the positive and negative controls to give percent growth at each concentration. The MIC was defined as the lowest concentration that gave ≤10% growth. When testing the MIC shift that occurs in the absence of WTAs, 0.4 μg $mL^{-1}$ tunicamycin was added to the media.

**WTA Extraction and PAGE Analysis Protocol.** WTAs were isolated from 5–10 mL cultures of *S. aureus* stationary-phase cells grown in TSB overnight at 30 °C in the presence of varying concentrations of tunicamycin. Tunicamycin stocks were made and diluted in DMSO and then added to the media to give 1% (v/v) final DMSO concentration. WTAs were extracted and visualized as previously reported (*16*).

**Growth Curves in the Presence of Tunicamycin.** Tunicamycin stocks were made and diluted in DMSO, and aliquots (1.5 μL) were added in duplicate to the wells of a 96-well plate. Overnight cultures of *S. aureus* strains were diluted 1/200 in fresh TSB, and 150 μL aliquots were added to the wells to give 1% (v/v) final DMSO concentration. The plate was then incubated at 30 °C (with shaking), and the optical density of each well was measured every 30 min for 7 h.

**Checkerboard Analyses of Tunicamycin and β-Lactams in MRSA Strains.** Stock solutions of tunicamycin were diluted 2-fold in DMSO. Aliquots (1.5 μL) were then transferred to the wells of a 96-well plate so that the concentrations varied along the rows. Solutions of β-lactams were made in $dH_2O$ and aliquotted (1.5 μL) into the wells of the 96-well plate so that dose responses ran down the columns. Stationary phase cultures of bacteria were diluted 1/1000 in fresh TSB, and 150 μL aliquots were dispensed into all wells. The plates were then covered and incubated at 30 °C for 16−24 h.

**COL Kill Curve in the Presence of Methicillin and Tunicamycin.** An overnight culture of COL was diluted 1/500 into fresh TSB. The starter culture was enumerated and split into three tubes. Methicillin (400 μg $mL^{-1}$) was added to one tube, and tunicamycin (0.4 μg $mL^{-1}$) was added to another. These cultures were shaken at 30 °C for 3 h, the tunicamycin-treated culture was split into two tubes, and methicillin was added to one of these. The cultures were shaken at 30 °C, and colony counts were taken each hour for an additional 9 h.

**Growth Conditions for Electron Microscopy Samples.** Overnight cultures of *S. aureus* were diluted 1/50 into 5 mL of fresh TSB and shaken at 30 °C to OD ~0.3. Compounds were added to the cultures, which continued to shake at 30 °C until harvesting. Samples were spun down (7500 × *g*; 8 min) each hour for 4 h. Pellets were resuspended in 0.25 mL of TSB, and 0.25 mL of fixative solution were then added to the sample. After 30 min at RT, the fixed samples were spun down and submitted to the Harvard Medical School EM Facility for processing. See Supporting Information for further details.

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*Note Added in Proof:* Atilano *et al.* have reported that *S. aureus* TarO (TagO) localizes to the septum and that ongoing WTA synthesis is required for the correct localization of PBP4. See: Atilano, M. L., Pereira, P. M., Yates, J., Reed, P., Veiga, H., Pinho, M. G., and Filipe, S. R. (2010) Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus, Proc. Natl. Acad. Sci. U.S.A.,* doi: 10.1073/pnas.1004304107.

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

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