



Revisiting the Role of VraTSR in *Staphylococcus aureus* Response to Cell Wall-Targeting Antibiotics

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ABSTRACT Exposure of *Staphylococcus aureus* to cell wall inhibitors leads to the activation of the VraTSR three-component sensory regulatory system. This system is composed of VraS, a membrane histidine kinase; VraR, its cognate response regulator, and VraT, a protein required for the full activity of VraTSR. The exact function of VraT remains mostly uncharacterized, although it has been proposed to detect the unknown stimulus sensed by the VraTSR system. Here, we elucidate the topology of VraT, showing that its C-terminal domain is extracellular. We also demonstrate that the signal sensed by VraTSR is not an intermediate in the peptidoglycan synthesis pathway, as previously suggested. Instead, the specific inhibition of the penicillin-binding protein (PBP)2 leads to strong activation of the system.

IMPORTANCE The Gram-positive bacterial pathogen *Staphylococcus aureus* is currently the second most frequent cause of global deaths associated with antibiotic resistance. Its response to cell wall-targeting antibiotics requires the VraTSR three-component system, which senses cell wall damage. Here, we show that the signal sensed by VraTSR is not an intermediate in the peptidoglycan synthesis pathway, as previously suggested. Instead, the specific inhibition of the penicillin-binding protein (PBP)2, the major peptidoglycan synthase in *S. aureus*, leads to strong activation of the system. Identifying the exact cell wall damage signal is key to fully understanding the response of *S. aureus* to cell wall-targeting antibiotics.

KEYWORDS *Staphylococcus aureus*, antibiotic resistance, peptidoglycan synthesis, two-component system

Staphylococcus aureus is a Gram-positive bacterial pathogen responsible for nosocomial and community-acquired infections worldwide. Methicillin-resistant *S. aureus* (MRSA) strains in particular are currently the second most frequent cause of global deaths associated with antibiotic resistance (1). *S. aureus* can cause a variety of infections in the human body, ranging from mild skin infections to life-threatening diseases (2). The versatility of this pathogen is partially due to its capacity to cope with changing environmental conditions, with adaptative responses coordinated by its 16 two-component systems (TCS) (3).

Bacterial TCS sense various environmental cues, including different chemical and physical stimuli such as ions, gases, antibiotics, osmotic pressure, and metabolites (4, 5). This allows bacterial cells to mount the appropriate response via changes in metabolism, cell division, antibiotic resistance, and pathogenicity, among other functions (6).

Among the several TCS present in *S. aureus*, the vancomycin resistance-associated regulatory system, also known as VraTSR, coordinates the bacterial response to cell wall (CW) synthesis disruption (7). VraTSR was initially identified due to its role in vancomycin resistance. However, it is also involved in the responses to several other compounds which target CW synthesis (8–10). Accordingly, disrupting this system increases the susceptibility of *S. aureus* toward several CW-targeting compounds (8).

VraTSR is encoded by the *vraUTSR* operon (7). VraS is a membrane histidine kinase belonging to the intramembrane-sensing histidine kinase (IMHK) family, since it is predicted to lack an extracellular sensing domain, which is characteristic of most histidine kinases (7, 11, 12).

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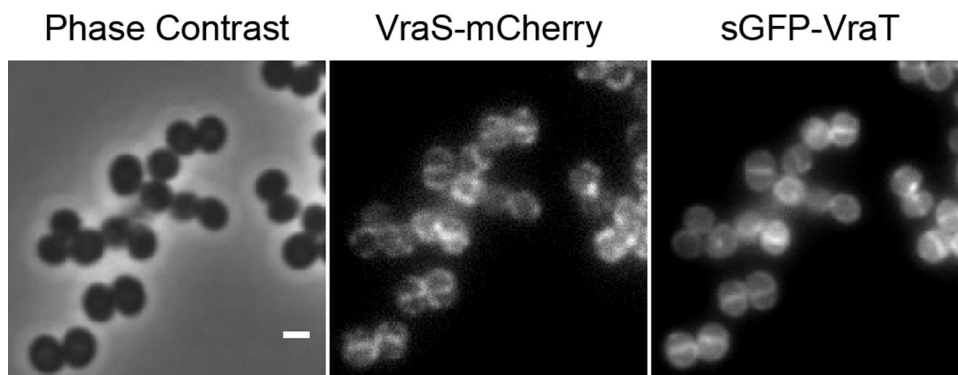


FIG 1 VraT and VraS localize at the cell membrane. Phase contrast (left) and epifluorescence microscopy images of *S. aureus* COL expressing a mCherry-tagged derivative of VraS (middle) and a sGFP-tagged derivative of VraT (right). The VraTSR system was induced by incubating the cells for 60 min with $0.5 \mu\text{g} \cdot \text{mL}^{-1}$ of CDFI, an inhibitor of the lipid II flippase MurJ. Scale bar 1 μm .

Upon detecting a still unknown stimulus, presumably resulting from *S. aureus* exposure to CW-targeting compounds, VraS undergoes autophosphorylation at a conserved histidine residue, likely Histidine 156 (11, 13). VraR is the cognate response regulator that is phosphorylated by VraS (11). Phosphorylated VraR dimerizes and binds to its own *vraUTSR* promoter, as well as to the promoters of several other genes, activating or repressing their transcription (11, 14). These genes, which constitute the VraR regulon, also known as the cell wall stress stimulon (CWSS) (15), belong to different functional categories, including lipid and carbohydrate metabolism, DNA replication and repair, and importantly, cell envelope biogenesis. The latter encompasses genes involved in peptidoglycan biosynthesis, such as *pbpB*, encoding the major *S. aureus* peptidoglycan synthase penicillin-binding protein 2 (PBP2); *mgt*, encoding a monofunctional glycosyltransferase; and *murZ*, which encodes a UDP-*N*-acetylglucosamine enolpyruvyl transferase (8, 9).

The role of VraT is less clear. Boyle-Vavra et al. have proposed that VraT (previously named YvqF) is the protein which detects the unknown stimulus sensed by the VraTSR system, promoting autophosphorylation of VraS, through a still-elusive mechanism (7). Accordingly, deletion of *vraT* affects the resistance of *S. aureus* to several CW-targeting antibiotics, similarly to what was observed for *vraS* and *vraR* deletion mutants (7, 16). Deletion of *vraT* also impairs VraTSR-dependent activation of the CWSS, showing its relevant role in this process (7, 16). VraT is predicted to be a membrane protein, described as interacting with VraS, but not VraR (7, 16). Its structure and topology are not known, and therefore the cellular localization of the C-terminal region, speculated to be the sensor domain, is yet to be determined (7, 16).

In this work, we aimed to unveil the identity of the stimulus responsible for VraTSR activation, specifically to determine whether this stimulus was an intermediate in the peptidoglycan synthesis pathway that would accumulate, or be depleted, in the presence of various CW-targeting antibiotics.

RESULTS

VraT is a membrane-localized protein with an extracellular C-terminal domain. The role of VraT has remained elusive, but it has been suggested to be involved in stimulus perception of the VraTSR regulatory system (7). We confirmed that both VraT and VraS localize to the membrane by using fluorescent fusions to both proteins (Fig. 1). This result indicates that, as expected, CW damage is perceived either at the cell surface or at the cell membrane.

Given that VraS, initially thought to be the sensor of the VraSR two-component system, does not display a clear extracellular sensing domain, characteristic of most histidine kinases, Boyle-Vavra et al. suggested that VraT was responsible for perception of CW damage on the outer surface of the cell (7, 12). However, the topology of VraT remained unclear, with different authors suggesting that its C-terminal domain lies intracellularly or extracellularly (7, 16). To examine the topology of VraT, we fused the *phoB* gene, encoding staphylococcal alkaline

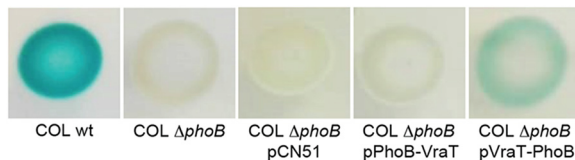


FIG 2 C-terminal domain of VraT is extracellular. VraT topology was assessed by fusing the *phoB* gene to *vraT* at either end. The *phoB* fusions to *vraT* were expressed from the pCN51 plasmid, under the control of a cadmium-inducible promoter, in the background of a *phoB* deletion mutant (COL Δ *phoB*). An empty pCN51 plasmid was also introduced into COL Δ *phoB* (COL Δ *phoB* + pCN51) and used as a negative control. The wild-type strain COL was used as positive control. The strains were grown on plates containing cadmium chloride and chromogenic BCIP (5-bromo-4-chloro-3-indolyl phosphate), a substrate which, when processed by the alkaline phosphatase PhoB, an enzyme active only in the extracytoplasmic environment, gives a blue coloration to the colonies (41). Only COL and COL Δ *phoB* carrying the *vraT-phoB* fusion (COL Δ *phoB* + pVraT-PhoB) showed alkaline phosphatase activity, indicating that the C-terminal domain but not the N-terminal domain of VraT faces the extracytoplasmic environment.

phosphatase, lacking its native export signal peptide, to either end of *vraT* and evaluated the enzymatic activity, as previously described (17). The alkaline phosphatase PhoB is active only in the extracytoplasmic environment, where it can process the chromogenic alkaline phosphatase substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate), generating a blue color. As shown in Fig. 2, only the PhoB C-terminal fusion to VraT showed some PhoB activity, indicating that the C-terminal domain of VraT is extracellular, while the N-terminal region is intracellular. This topology is compatible with a sensor function for the C-terminal domain of VraT.

VraTSR does not sense an intermediate in peptidoglycan synthesis. CW synthesis is a multi-enzymatic, sequential process that leads to the formation of the lipid II molecule (Fig. 3a) (18). Lipid II is subsequently flipped to the extracellular space and inserted in the peptidoglycan (PG) mesh (18). Thus, the accumulation or depletion of an intermediate molecule in this enzymatic pathway, particularly lipid II, would be a good candidate for the signal sensed by VraTSR. We tested the effects of various antibiotics blocking the initial or final steps of the PG synthesis pathway (Fig. 3a) and determined that in all cases, the VraTSR response was triggered (Fig. 3b and Fig. S1 in the supplemental material), confirming previous work (10). This was done by incubating COL P_{vra}-sGFP cells, where GFP expression is driven by the *vraTSR* promoter, with 1 \times the MICs of different CW-targeting antibiotics for 60 min and subsequently analyzing the fluorescence of the cells by fluorescence-activated cell sorting (FACS). Antibiotics blocking the early stages of CW synthesis, like fosfomycin, are expected to inhibit the formation of all subsequent lipid precursors, including lipid II, while antibiotics blocking the late steps of this process, like vancomycin, were previously shown to accumulate lipid II (19, 20). Considering that these antibiotics produce opposite effects on PG precursor levels, but all result in the activation of the VraTSR response, it seems unlikely that VraTSR is sensing the accumulation or depletion of lipid-linked PG precursors.

Inhibition of transglycosylase activity induces VraTSR. Given that we were unable to identify one specific CW synthesis precursor as the signal that triggers the VraTSR system, we decided to investigate whether the system was triggered by lack of activity of a particular enzyme involved in CW synthesis. We started by focusing on the late stages of CW synthesis performed by PBPs (1 to 4), the exogenous PBP (PBP2A), and the monofunctional glycosyltransferases MGT and SgtA (21). Two of these enzymes, the bifunctional transpeptidase-transglycosylase PBP2 and the monofunctional transglycosylase MGT, have their genetic expression upregulated by the response regulator VraR as part of the CWSS (8, 9). Deletion of the gene encoding PBP2, but not of those encoding other PBPs, led to the activation of VraTSR (Fig. 4). PBP2 is the only bifunctional PBP in *S. aureus*, while the remaining PBPs display monofunctional transpeptidase activity (21). These results are in line with previous work showing that decreasing levels of *pbpB* (encoding PBP2) transcription lead to increasing levels of *vraTSR* transcripts (22).

To explore the mechanism behind PBP2-dependent VraTSR activation, we evaluated whether the delocalization of PBP2 from the division septum was the signal detected by VraTSR. PBP2 is recruited to the division septum by binding to its substrate, lipid II (23), and the presence of CW synthesis inhibitors causes PBP2 delocalization (Fig. S2 in the supplemental

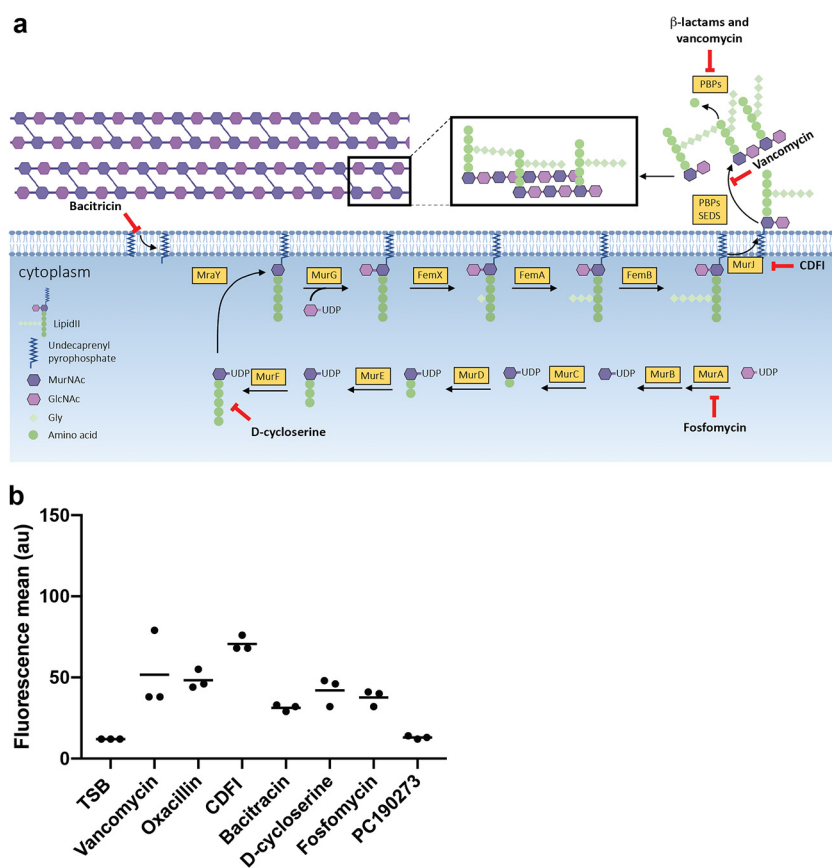


FIG 3 *VraTSR* responds to various cell wall (CW)-targeting antibiotics (a) Schematic representation of peptidoglycan synthesis pathway, indicating the targets of cell wall active antibiotics. Fosfomycin inhibits the enzyme MurA (UDP-*N*-acetylglucosamine-3-enolpyruvyl transferase) that performs the addition of phosphoenolpyruvate to UDP-*N*-acetylglucosamine (GlcNAc), generating UDP-*N*-acetyl-muramic acid (UDP-MurNAc) (42). *D*-Cycloserine inhibits the addition of *D*-alanine to the peptidoglycan precursor by inhibiting *D*-alanine:*D*-alanine ligase A and alanine racemase (43). CDFI inhibits MurJ (44), the lipid II flippase. Bacitracin blocks dephosphorylation and recycling of the lipid carrier undecaprenyl pyrophosphate, preventing the membrane steps of peptidoglycan synthesis (45). Glycopeptide antibiotics, such as vancomycin, bind to the *D*-Ala-*D*-Ala motif of lipid II, blocking the access of enzymes performing transglycosylase and/or transpeptidase activity (46). β -Lactam antibiotics, such as oxacillin, mimic the *D*-Ala-*D*-Ala end of lipid II stem peptide, forming an acyl-enzyme complex with PBPs, inhibiting their activity (47). (b) COL P_{vra}-sGFP cells, which express green fluorescent protein (GFP) under the control of the *vraTSR* promoter, were incubated with $1 \times$ MIC of different CW-targeting antibiotics for 60 min prior to analysis by fluorescence-activated cell sorting (FACS). All tested CW-targeting antibiotics activated the *VraTSR* system independently of the blocked step of the PG synthesis pathway. Incubation with the FtsZ inhibitor PC190273, an antibiotic that does not target CW synthesis, was included as a negative control. TSB indicates growth in the absence of antibiotics. $N = 5,000$ cells for each replicate; experiments were done in triplicate. Each point represents the median of one FACS experiment.

material) in agreement with previous observations (23, 24). To test this hypothesis, we incubated cells expressing a fluorescent derivative of PBP2 (strain BCBPM073) and cells expressing green fluorescent protein (GFP) under the control of the *vraTSR* promoter (COL P_{vra}-sGFP) with increasing concentrations of vancomycin. Interestingly, we observed that PBP2 delocalized with concentrations as low as $1 \mu\text{g} \cdot \text{mL}^{-1}$ of vancomycin, while *VraTSR* remained untriggered at that concentration (Fig. 5). In the experimental settings tested, we could only observe *VraTSR* activation when we incubated the cells with $10 \mu\text{g} \cdot \text{mL}^{-1}$ of vancomycin, a concentration 10-fold higher than that required to delocalize PBP2 from the septum (Fig. 5), indicating that PBP2 delocalization alone is not the trigger for the *VraTSR* system.

Because PBP2 delocalization was not the trigger for *VraTSR* activation, we further explored whether lack of this protein or of one of its two enzymatic activities (transpeptidation and transglycosylation) activated the *VraTSR* system. For that purpose, the strain lacking PBP2 and containing the *vraTSR* GFP reporter (COL Δ *pbpB*_P_{vra}-sGFP) was complemented with plasmid-encoded PBP2, as well as plasmid-encoded alleles of PBP2, with point mutations to

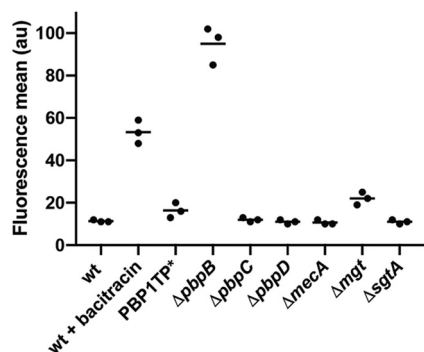


FIG 4 Depletion of PBP2, but not of the remaining CW synthesis enzymes, leads to VraTSR activation. *S. aureus* mutants lacking different CW synthesis enzymes, PBP2 ($\Delta pbpB$), PBP3 ($\Delta pbpC$), PBP4 ($\Delta pbpD$), PBP2A ($\Delta mecA$), MGT (Δmgt), or SgtA ($\Delta sgtA$), or encoding a transpeptidase mutant of the essential PBP1 (PBP1TP*) and expressing GFP under the control of the *vraTSR* promoter, were analyzed by FACS. Only the cells lacking PBP2 ($\Delta pbpB$) showed strong VraTSR activation, while the Δmgt mutant showed low activation levels. Control experiments, where COL P*vra*-sGFP cells were grown in the absence (wt) or presence of bacitracin (wt + bacitracin), which induces the VraTSR response, were performed as negative and positive controls, respectively. $N = 5,000$ cells for each replicate; experiments were done in triplicate. Each point represents the median of one FACS experiment.

individually ablate transglycosylation, transpeptidation, or both activities simultaneously. When COL $\Delta pbpB$ _P*vra*-sGFP was complemented with an empty plasmid, VraTSR remained activated (Fig. 6), in agreement with the data shown in Fig. 4 for the *pbpB* mutant. Complementation with the double mutant of *pbpB* had similar results, indicating that it is the lack of PBP2 activity and not of the protein itself that signals CW damage. As expected, complementation with the wild-type *pbpB* allele lowered *vraTSR* expression to levels equivalent to those of the parental strain COL P*vra*-sGFP. We then focused on which PBP2 activity was relevant for VraTSR activation. Complementation with the construct coding for the glycosyltransferase inactive PBP2^{E114Q} resulted in induced VraTSR, suggesting that a lack of this activity could be key to activating the system. However, complementation with the transpeptidase-inactive PBP2^{S398G} also induced VraTSR, suggesting that either both activities are required to turn off the system or a mutation in one domain may compromise the activity of the other. In fact, the MIC of moenomycin, an antibiotic that inhibits transglycosylase activity (25), for the mutant complemented with the PBP2 lacking transpeptidase activity, showed increased susceptibility toward this antibiotic compared with COL P*vra*-sGFP, although not to the same level as the strain complemented with the PBP2 transglycosylase mutant (Fig. 6b).

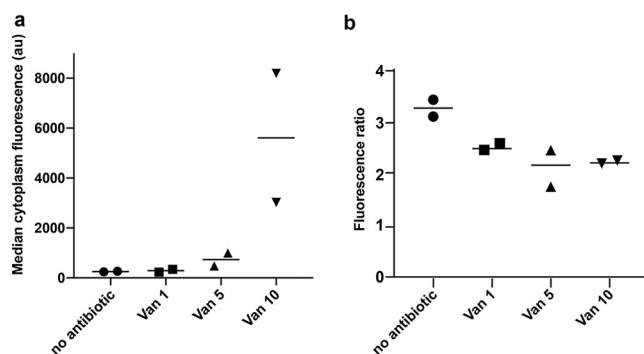


FIG 5 PBP2 delocalization does not correlate with VraTSR activation. Strains COL P*vra*-sGFP and BCBPM073 (expressing a GFP fluorescent derivative of PBP2) were both incubated in the presence of increasing vancomycin concentrations (Van 0, 1, 5, and 10 $\mu\text{g} \cdot \text{mL}^{-1}$) for 45 min. COL P*vra*-sGFP cells were then stained with DNA dye Hoechst 33342 to differentiate the two populations and mixed with BCBPM073 cells just prior to imaging on the same microscope slide. (a) GFP fluorescence of COL P*vra*-sGFP cells, showing that VraTSR activation occurs only in the presence of 10 $\mu\text{g} \cdot \text{mL}^{-1}$ of vancomycin. $N > 277$ cells for each condition in each replicate. (b) PBP2 septal enrichment was evaluated in BCBPM073 cells by measuring the fluorescence ratio of GFP-PBP2 fluorescence at the septum versus at the cell periphery, both corrected for background fluorescence. PBP2 delocalized from the septum at the lowest vancomycin concentration tested. $N =$ between 62 and 90 for each condition in each experiment; experiments performed in duplicate.

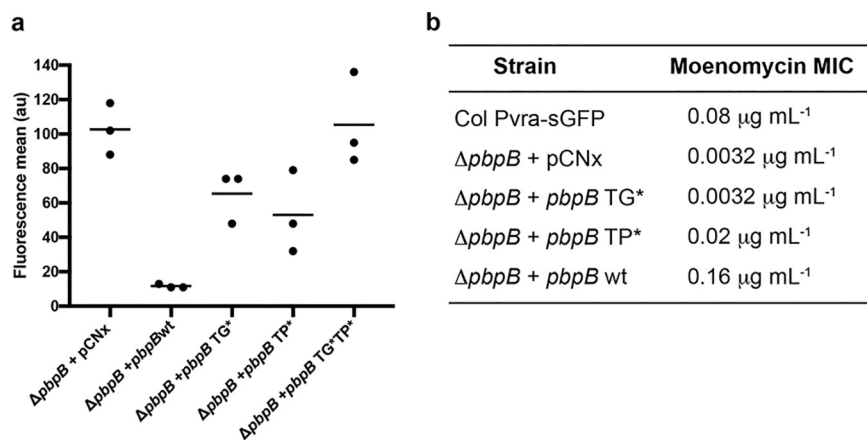


FIG 6 PBP2 transglycosylase and transpeptidase activities are both important for preventing *VraTSR* activation. (a) Strain COL $\Delta pbpB$ -P_{vra}-sGFP was complemented with plasmids encoding either the wild-type allele of *pbpB* ($\Delta pbpB + pbpB wt$) or mutated alleles encoding a transglycosylase inactive PBP2^{E114Q} ($\Delta pbpB + pbpB TG^*$), a transpeptidase inactive PBP2^{S398} ($\Delta pbpB + pbpB TP^*$), or a double mutant PBP2^{E114Q S398} ($\Delta pbpB + pbpB TG^*TP^*$). A control complemented with an empty vector ($\Delta pbpB + pCNx$), lacking PBP2, was also included. The *VraTSR* system was induced in the absence of PBP2 or in the presence of any of the tested PBP2 mutants. $N = 5,000$ cells for each experiment; experiments performed in triplicate. Each point represents the median of one FACS experiment. (b) Moenomycin MICs for strains from panel a, as well as that for the control strain COL P_{vra}-sGFP.

This indicates that the mutation in the transpeptidase domain of PBP2 may partially impair transglycosylation activity, possibly because the two subunits perform a consecutive enzymatic process in which the transpeptidase accepts the substrate from the transglycosylase, so the transglycosylase activity may be impaired if the transpeptidase is inactive.

Taken together, these results suggest that the inhibition of transglycosylase activity seems to be key in triggering the *VraTSR* regulatory system. We cannot discard a role for inhibition of transpeptidase activity, but we favor the former hypothesis, since (i) the depletion of the other PBPs (monofunctional transpeptidases) did not activate *VraTSR* and (ii) COL Δmgt , a strain lacking the monofunctional transglycosylase MGT, showed *VraTSR* activation, albeit to low levels.

DISCUSSION

Two-component and three-component systems (TCS) are incredibly versatile, capable of detecting a wide variety of stimuli (26). Among the 16 two-component systems described in *S. aureus*, *VraTSR* is responsible for detecting CW damage, although the exact signal that is detected remains unknown (7, 9). Other *S. aureus* TCS, GraXSR and BraSR, also respond to damage inflicted on the CW (27, 28), with additional partners being required for full activity. In the GraXSR system, which responds to antimicrobial peptides (CAMPs), the sensing mechanism depends on the ABC transporter *VraFG*, encoded by an operon located directly downstream of *graXRS* (27, 29). CAMPs are sensed by *VraFG*, and the signal is transduced to GraS through a possible interaction between *VraG* and GraS (27). BraSR, which responds to bacitracin, upregulates the transcription of two operons encoding ABC transporters, *braDE* and *vraDE*, which act as sensing partner and detoxification module, respectively (28). Interestingly, both GraXSR and BraSR have IMHKs, a feature shared with the *VraTSR* system (27). The putative sensing partner in the *VraTSR* regulatory system has been proposed to be the membrane protein *VraT*, although its structure and function remain undetermined (7). Here, we have shown that *VraT* localizes at the cell membrane, like the histidine kinase *VraS*, and that its C-terminal domain is extracellular. Since *VraS* lacks a typical histidine kinase extracellular sensing domain, the signal detection by *VraTSR* may be performed by *VraT*, as previously suggested (7).

Given the importance of *VraTSR* in the antibiotic resistance of MRSA strains, there is great interest in identifying the signal sensed by this system. It was clear early on that

the system does not directly sense antibiotic molecules, given that structurally different molecules which target CW synthesis are capable of inducing *VraTSR*. The best candidate so far for the CW damage signal has been the accumulation or depletion of the peptidoglycan precursor lipid II (30). This molecule is synthesized on the inner side of the cytoplasmic membrane and then flipped to the outer side of the membrane (18). If accumulation of lipid II triggered the *VraTSR* system, one would expect its activation in the presence of antibiotics such as vancomycin, which lead to lipid II accumulation (20), but not in the presence of antibiotics such as fosfomycin or D-cycloserine, which lead to its depletion. The reverse would be expected if depletion of lipid II was the CW damage signal. However, all tested CW-targeting antibiotics triggered the *VraTSR* system, indicating that neither the accumulation nor the depletion of any peptidoglycan synthesis precursor is likely to correspond to this signal.

A common effect of all CW-targeting antibiotics tested is the depletion of the substrate for PBPs. *S. aureus* has four native PBPs (PBPs 1 to 4), with one extra PBP (PBP2A) from an exogenous origin, with low affinity to beta-lactam antibiotics, which is present in MRSA strains (21). PBP2 is the only bifunctional PBP, with both transglycosylase and transpeptidase activities, while the others are monofunctional TPases (31–36). Two *S. aureus* monofunctional PBPs, namely, the essential PBP1 and the nonessential PBP3, which only have transpeptidase activity, interact with cognate shape, elongation, division and sporulation (SEDS) transglycosylases, FtsW and RodA, respectively, to synthesize PG (34). Monofunctional glycosyltransferases are also present in *S. aureus*, namely, MGT and SgtA, both of which are nonessential for cell viability (37, 38). Here, we showed that when we individually depleted various PG synthases, only the absence of PBP2 led to strong *VraTSR* activation, while lack of MGT lead to low-level activation of *VraTSR*. This is in accordance with previous work showing that reducing the levels of *pbpB* transcription led to higher levels of *vraTSR* transcripts (22). PBP2 is the only PBP whose expression is directly regulated by *VraR*, a feature shared with MGT (9, 39). This indicates that PBP2 activity is crucial to maintain the *VraTSR* system in the OFF state. A second common effect of all CW-targeting compounds tested, besides depletion of substrate for PBPs, is the delocalization of PBP2 from the division septum (Fig. S2) (23, 24). Therefore, one possible mechanism mediating the role of PBP2 in *VraTSR* activation could be a direct interaction between this protein and *VraT/VraS*, disrupted upon PBP2 delocalization in the presence of CW-targeting compounds. However, this is unlikely to be the case, because we showed that PBP2 delocalization does not correlate with *VraTSR* activation: the former was observed in the presence of low ($1 \mu\text{g} \cdot \text{mL}^{-1}$) concentrations of vancomycin, which were insufficient for *VraTSR* activation (see Fig. 5). In contrast, inactivation of either the transglycosylase or transpeptidase domain of PBP2 lead to a strong activation of the *VraTSR* system. Unfortunately, we could not pinpoint which of the two activities had a major role, because inactivation of the transpeptidase domain of PBP2 impaired the activity of the transglycosylase domain, leading to an increased susceptibility to the transglycosylase inhibitor moenomycin. It is possible that processivity of the transglycosylase activity of PBP2 is impaired if the resulting glycans cannot be incorporated into the peptidoglycan mesh via transpeptidation. We favor the hypothesis that it is the decrease of transglycosylase activity that signals cell wall damage because removal/inactivation of the other transpeptidases (PBP1, PBP3, and PBP4) did not result in *VraTSR* activation; in particular, removal/inactivation of PBP4, whose absence leads to a major decrease in peptidoglycan cross-linking (35). Additionally, lack of MGT, and not of any of the other peptidoglycan synthases tested, also led to *VraTSR* activation, albeit to a lower level. A decrease in PBP2 transglycosylase activity leads to shorter glycans (40) and therefore to an increase in the number of glycan strand extremities, which could be sensed by *VraTSR*. Alternatively, a reduction in transglycosylase activity without concomitant reduction of the activity of peptidoglycan hydrolases could lead to an increase in the concentration of a peptidoglycan hydrolysis product that could be sensed as CW damage by *VraTSR*. Identifying the exact CW damage signal is key to fully understanding the response of MRSA strains to beta-lactam antibiotics.

MATERIALS AND METHODS

Bacterial growth conditions. The plasmids and strains used in this study are listed in Tables S1 and S2 and their construction is described in the supplemental material. *S. aureus* strains were grown in tryptic soy broth (TSB, Difco) with aeration at 37°C or on tryptic soy agar (TSA, Difco) at 30°C or 37°C. For microscopy experiments, overnight cultures of *S. aureus* strains were diluted 1:200 in TSB medium and allowed to grow at 37°C until an optical density at 600 nm (OD_{600}) of approximately 0.5. Cells were then harvested and resuspended in the same medium. *Escherichia coli* strains were grown in Luria-Bertani broth (LB, Difco) with aeration, or on LB agar (LA, Difco) at 30°C. The culture medium was supplemented with the appropriate antibiotics ($100 \mu\text{g} \cdot \text{mL}^{-1}$ ampicillin, $10 \mu\text{g} \cdot \text{mL}^{-1}$ erythromycin, $10 \mu\text{g} \cdot \text{mL}^{-1}$ chloramphenicol, or $50 \mu\text{g} \cdot \text{mL}^{-1}$ of both kanamycin and neomycin; Sigma-Aldrich), with $100 \mu\text{g} \cdot \text{mL}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; VWR) or cadmium chloride ($0.1 \mu\text{M}$, Sigma-Aldrich), when required. Cell wall-targeting antibiotics were used at their MICs: $2 \mu\text{g} \cdot \text{mL}^{-1}$ for vancomycin, $800 \mu\text{g} \cdot \text{mL}^{-1}$ for oxacillin, $1.5 \mu\text{g} \cdot \text{mL}^{-1}$ for CDFI [2-(2-chlorophenyl)-3-[1-(2,3-dimethylbenzyl)piperidin-4-yl]-5-fluoro-1H-indole], $40 \mu\text{g} \cdot \text{mL}^{-1}$ for bacitracin, $125 \mu\text{g} \cdot \text{mL}^{-1}$ for D-cycloserine, $300 \mu\text{g} \cdot \text{mL}^{-1}$ for fosfomycin, and $1 \mu\text{g} \cdot \text{mL}^{-1}$ for PC190273.

MIC assays. MICs of relevant antimicrobial compounds were determined by broth microdilution in sterile 96-well plates. Series of 2-fold dilutions of each compound were performed in TSB. Cultures of *S. aureus* strains and mutants were added at a final density of $\sim 5 \times 10^5 \text{ CFU} \cdot \text{mL}^{-1}$ to each well. In each plate, some wells were not inoculated, for sterility control, and cell viability was assessed in wells with TSB to which no compound was added. Plates were incubated at 37°C. Endpoints were assessed visually after 24 and 48 h and the MIC was determined as the lowest compound concentration that inhibited growth. All assays were performed in triplicate.

Fluorescence-activated cell sorting analysis. Overnight TSB cultures of *S. aureus* strains were diluted 1:200 in TSB and grown at 37°C to exponential growth phase (OD_{600} of 0.5 to 0.7). The appropriate antibiotic was then added, and cells were incubated for 1 h. After antibiotic incubation, cells were collected by centrifugation, washed once with PBS (phosphate-buffered saline, NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM), and resuspended in PBS. Samples were filtered with a 40- μm pore strainer and diluted 1:50 in PBS. Flow cytometry data were collected on a S3e Cell Sorter (Bio-Rad) using a target flow rate of 500 events per second and collecting 5,000 events for each sample. A 100-mW 488-nm laser line was used for excitation, with amplification settings of 350 forward scattering (FSC) and 950 (FL1, 525/30 nm). Acquisition was triggered by forward scattering with a threshold of 1.15.

***S. aureus* imaging by fluorescence microscopy.** For fluorescence microscopy experiments, *S. aureus* cultures were grown to mid-exponential phase (OD_{600} of 0.5 to 0.7) and 1 mL was collected by centrifugation. Cells were suspended in 30 μL of PBS and 1 μL was placed on a thin layer of agarose (1.2% in PBS). When incubation with antibiotics was required prior to imaging, *S. aureus* cells were grown to an OD_{600} of 0.3 to 0.4 at which point appropriate compounds were added. Cells were further incubated for 60 min at 37°C, before being collected by centrifugation.

To evaluate septal enrichment when studying protein localization, we determined the fluorescence ratio (FR) calculated as the ratio of the median fluorescence of the 25% brightest pixels of the septum versus median fluorescence at the cell periphery, both corrected for background fluorescence. To measure FRs and cytoplasmic fluorescence in two different strains (COL PvrA-sGFP and BCBPM073) incubated for 45 min with increasing concentrations of vancomycin (0, 1, 5 or $10 \mu\text{g mL}^{-1}$), strains were imaged on the same microscopy slide. For this, COL PvrA-sGFP cells were labeled with DNA dye Hoechst 33342 ($1 \mu\text{g mL}^{-1}$), both cultures were washed with TSB, and then the two cultures were mixed prior to visualization by epifluorescence microscopy.

Microscopy was done using a Zeiss Axio Observer microscope with a Plan-Apochromat $100\times/1.4$ oil Ph3 objective. Images were acquired with a Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices).

Determination of VraT topology using PhoB fusions. Overnight cultures of wild-type COL, COL Δ phoB, COL Δ phoBpCN51, COL Δ phoBpVraT-PhoB and COL Δ phoBpPhoB-VraT were diluted in TSB to a final OD_{600} of 0.05 and grown to mid-exponential phase ($OD_{600} \sim 0.5$). Subsequently, 1 mL of culture was harvested by centrifugation and resuspended in the same volume of TSB. Serial 10-fold dilutions of each strain were made and 20 μL of 10^0 , 10^{-2} , 10^{-4} , and 10^{-6} dilutions were plated onto tryptic soy agar plates containing BCIP (Sigma-Aldrich), supplemented with erythromycin $10 \mu\text{g} \cdot \text{mL}^{-1}$ and 2 μM cadmium chloride (Sigma-Aldrich), when appropriate. Plates were incubated at 37°C for 24h.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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