Revisiting the role of VraTSR in *Staphylococcus aureus* response to cell wall targeting antibiotics

Supplemental Information

Pedro B. Fernandes, Patricia Reed, João M. Monteiro and Mariana G. Pinho

Supplemental Methods

Construction of *S. aureus* strains

Sequence of primers used for strain construction as listed in Supplemental Table 3.

To construct the *vraTSR* promoter fusion, an 814-bp DNA fragment containing the *vraTSR* promoter region was amplified from COL genomic DNA using primers P vraSR_P1_KpnI and P vraSR_P2_New_XhoI, digested with KpnI and XhoI, and cloned into KpnI/XhoI digested pFAST3, upstream of sfgfp-p7, resulting in plasmid pP vra-pFAST3, confirmed by DNA sequencing. pP vra-pFAST3 was electroporated into the *S. aureus* RN4220 strain and integrated into the chromosome at the *vraTSR* promoter site by homologous recombination, as confirmed by PCR and sequencing; the resulting strain was named RN P vra-sGFP. Strain COL P vra-sGFP was constructed by transducing the integrated plasmid pP vra-pFAST3 from RN P vra-sGFP into COL using phage80α, as previously described

To construct the *S. aureus ΔpbpB* null mutant, lacking PBP2, we amplified 1Kb DNA fragments from *S. aureus* COL genomic DNA corresponding to the upstream (primers PBP2_KO-P1 and PBP2_KO-P2) and downstream (primers PBP2_KO-P13 and PBP2_KO-P4) regions of the *pbpB* gene. The resulting PCR products were joined by overlap PCR using primers PBP2_KO-P1 and PBP2_KO-P4. The overlap PCR product was digested with EcoRI and BamHI and cloned into the thermosensitive plasmid pMAD2, producing plasmid pHΔpbpB. The plasmid was sequenced and introduced into RN4220 by electroporation. Following electroporation, the plasmid was transduced into COL using phage 80α.
as previously described\textsuperscript{1}. Insertion and excision of pΔpbpB was performed as previously described\textsuperscript{2}, except the integration steps were performed at 37 °C instead of 43 °C, resulting in strain COLΔpbpB. Deletion of the target gene was verified by PCR and resulting strains were verified by whole genome sequencing.

To monitor vraTSR expression levels in the mutants lacking different enzymes involved in the last stages of peptidoglycan synthesis, pPvra-pFAST3 was transduced into the corresponding mutant strains, using phage 80α, with erythromycin selection, resulting in strains: ColPB1TP_Pvra-sGFP, COLΔpbpB_Pvra-sGFP, COLΔpbp3_Pvra-sGFP, COLΔpbpD_Pvra-sGFP, COLΔmgt_Pvra-sGFP, COLΔsgtA_Pvra-sGFP and COLΔmecA_Pvra-sGFP.

To investigate the mechanism behind PBP2-dependent VraTSR activation, COLΔpbpB_Pvra-sGFP was complemented with plasmids encoding different alleles of PBP2. For that, a 2214-bp fragment encompassing the wild type pbpB allele was amplified using primers PBP2muts_P1 and PBP2muts_P2 from S. aureus COL genomic DNA. The same pair of primers was used to amplify a fragment, coding for the transglycosylase-inactive PBP2\textsuperscript{E114Q} from COLTG42\textsuperscript{4} genomic DNA. These fragments were introduced into Smal-digested pCNX using a Gibson assembly cloning kit (NEB), originating plasmids pPBP2 and pPBP2TG. Transduction of these plasmid into COLΔpbpB_Pvra-sGFP gave COLΔpbpB_Pvra-sGFPpPBP2 and COLΔpbpB_Pvra-sGFPpPBP2TG*, respectively. To construct plasmid pCNX-pbp2TP, a full copy of pbpB allele encoding for PBP2\textsuperscript{S398G} (pbp2TP) was cloned into the pCNX plasmid, downstream of the cadmium inducible P\textsubscript{cad} promoter. The pbp2TP sequence was amplified from pMAD-pbp2TPbig which was constructed by amplifying two PCR fragments encompassing 1.7 kb upstream or downstream of nucleotide T1192 of pbpB using primers P1pMADpbp2TP/ P2pMADpbp2TP and P3pMADpbp2TP/ P4pMADpbp2TP, respectively. The two fragments were joined by overlap PCR using primers pair P1pMADpbp2TP and P4pMADpbp2TP, digested with BglII and Smal and cloned into pMAD, creating plasmid pMADpbp2TPbig. This plasmid contains the nucleotide exchanges T1192G and C1193G which switch the catalytic serine from the TP domain of PBP2 to a glycine (S398G) and a silent mutation (T1197C) that introduces a BamHI restriction site to facilitate screening
of clones. The pMAD-PBP2TPbig plasmid served as a template to amplify the full pbp2TP allele using primers P1pCNXpbp2 and P2pCNXpbp2. The resulting PCR fragment was cloned downstream of the Pcad promoter of pCNX plasmid, after digesting with Sall and KpnI, resulting in plasmid pCNX-pbp2TP. pCNX-pbp2TP was then transduced to COLΔpbpB_Pvra-sGFP, originating strain COLΔpbpB_Pvra-sGFPpPBP2TP*. To construct pBP2TGTP, the mutation coding for transglycosylase-inactive PBP2\textsuperscript{E114Q} was inserted into plasmid pCNX-pbp2TP, via site-directed mutagenesis, using primers E114Q fw and E114Q rev. The resulting plasmid was transduced to COLΔpbpB_Pvra-sGFP, giving strain COLpbpB_Pvra-sGFPpPBP2TG*TP*.

To localize VraT and VraS, an N-terminal fusion of VraT to the P7 variant of superfast GFP (sfGFP)\textsuperscript{5} and a C-terminal fusion of VraS to mCherry were constructed. A 409-bp fragment encoding the upstream region of vraT was amplified by PCR from S. aureus COL genomic DNA, using primers orf1\_fw\_EcoRI and orf1\_rev\_link\_sgfp. Primers sgfp\_fw\_link\_orf1 and sgfp\_rev\_link\_vraT were used to amplify gfp from pFAST3. A third 744-bp fragment containing vraT and a sequence encoding an 11 amino acid linker was amplified using primers vraT\_fw\_link\_sgfp and vraT\_rev\_BamHI. The fragments were joined by overlap PCR using the primers orf1\_fw\_EcoRI and vraT\_rev\_BamHI. The resulting fragment was digested with EcoRI and BamHI restriction enzymes and cloned into pMAD, giving pVraT\_GFP. The plasmid was sequenced and then electroporated into S. aureus RN4220 strain at 30 °C, using erythromycin and X-gal selection, and transduced to COL using phage 80α. Integration and excision of the plasmid into the chromosome was performed as previously described\textsuperscript{2}, giving COLsGFP\_VraT. An 862-bp fragment encoding VraS, excluding the stop codon, and a sequence encoding an 11 amino acid linker was amplified by PCR from S. aureus COL genomic DNA, using primers VraSCterfwd\_NcoI and VraSmchMLbwd. Primers mCherryMLfwd and mCherrybwd2 were used to amplify mCherry from pBCB4-Cherry. A third 860-bp fragment containing the downstream region of vraS was amplified using primers Vrasfwd3 and VraRbwd\_BamHI. The fragments were joined by overlap PCR using the primers mCherryMLfwd and VraRbwd\_BamHI. The resulting fragment was digested with Ncol and BamHI restriction enzymes and cloned into pMAD, giving
pVraS-mCherry. The plasmid was sequenced and then electroporated into *S. aureus* RN4220 strain at 30 °C, using erythromycin selection in the presence of X-gal, and transduced into COLsGFP_VraT, using phage 80α. Integration and excision of the plasmid into the chromosome was performed as described\(^2\), resulting in strain COLsGFP-VraTVraS-mCherry.

To determine the topology of VraT, PhoB fusions to VraT were made at both N- and C-terminal ends. For the N-terminal fusion to VraT a 1389-bp fragment encompassing *phoB* allele and a 5 amino acid linker was amplified using primers phoB_vraT_P1 and phoB_vraT_P2 from *S. aureus* COL genomic DNA. Another fragment with 748-bp with *vraT* coding sequence was amplified from the same genomic DNA, using primers phoB_vraT_P3 and phoB_vraT_P4. These fragments were introduced into Smal-digested pCN51 using a Gibson assembly cloning kit (NEB), originating plasmid pPhoB_VraT. For the C-terminal PhoB fusion to VraT, a 772-bp fragment with *vraT* coding sequence and encoding a 5 amino acid linker was amplified from *S. aureus* COL genomic DNA, using primers vraT_phoB_P1 and vraT_phoB_P2. Primers vraT_phoB_P3 and vraT_phoB_P4 were used to amplify a 1376-bp DNA fragment with *phoB* coding sequence, from the same genomic template. These fragments were introduced into Smal-digested pCN51 using a Gibson assembly cloning kit (NEB), originating plasmid pVraT-PhoB. Plasmids pCN51, pPhoB_VraT and pVraT-PhoB were transduced to COLΔ*phoB*, giving strains COLΔ*phoBpCN51*, COLΔ*phoBpPhoB-VraT* and COLΔ*phoBpVraT-PhoB*, respectively.
Supplementary Figure 1. Blocking early, middle or late stages of CW synthesis results in VraTSR activation. *S. aureus* cells expressing GFP under the control of the *vraTSR* promoter (COL P*vra*-sGFP) were incubated with 1x MIC of different CW targeting antibiotics for 60 mins prior to imaging by fluorescence microscopy. Cells from a control experiment, with no antibiotic, were always present on the same slide and those cells were labelled with DNA dye Hoechst 33342, to discriminate the two populations. Incubation with the FtsZ inhibitor PC190273, an antibiotic that does not target the CW synthesis, was also included as a control. N > 223 cells for each condition. Data represented in violin plots where the middle line represents the median and the other two lines the quartiles.
Supplementary Figure 2. PBP2 delocalizes in the presence of cell wall targeting antibiotics. COL *pbpB::sgfp-pbpB* (BCBPM073) cells were imaged by epifluorescence microscopy after incubation for 60 min with 1 x MIC of PC190273, (+PC, 1 µg mL$^{-1}$), fosfomycin (+Fosfo, 300 µg mL$^{-1}$), D-cycloserine (+D-cyc, 125 µg mL$^{-1}$), bacitracin (+Bac, 40 µg mL$^{-1}$), 2-(2-Chlorophenyl)-3-[1-(2,3-dimethylbenzyl)piperidin-4-yl]-5-fluoro-1H-indole (+CDFI, 1.5 µg mL$^{-1}$), oxacillin (+Oxa, 800 µg mL$^{-1}$) and vancomycin (+Van, 2 µg mL$^{-1}$). Incubation in TSB without antibiotics was used as negative control. In the presence of all antibiotics tested, septal enrichment of PBP2 is lost or decreased and the protein becomes dispersed over the membrane. Scale bar = 1µm
**Supplemental Tables**

**Supplementary Table 1. Plasmids used in this study**

| Plasmids        | Description                                                                 | Source or reference |
|-----------------|----------------------------------------------------------------------------|---------------------|
| pCN51           | Shuttle vector containing a cadmium inducible Pcad promoter; Amp^R^ Ery^R^   | 6                   |
| pCNX            | Shuttle vector containing a cadmium inducible Pcad promoter; Amp^R^ Kan^R^  | 7                   |
| pMAD            | *E. coli-S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; Amp^R^ Ery^R^ lacZ | 2                   |
| pBCB4-ChE       | *S. aureus* integrative vector for N- and C-terminal mCherry fusions; Amp^R^ Ery^R^ | 8                   |
| pFAST3          | *S. aureus* integrative vector that allows for C-terminal sGFP fusions; Amp^R^ Ery^R^ | 9                   |
| pVra-pFAST3     | *S. aureus* integrative vector with vraTSR promoter upstream of sgfp-p7   | This study          |
| pPBP2           | pCNX derivative containing *pbpB* under the control of Pcad promoter; Amp^R^ Kan^R^ | This study          |
| pPBP2TG         | pCNX derivative containing *pbpBTG*(E114Q) under the control of Pcad promoter; Amp^R^ Kan^R^ | This study          |
| pCNX-pbp2TP     | pCNX derivative containing *pbp2TP*(S398G) under the control of Pcad promoter; Amp^R^ Kan^R^ | This study          |
| pPBP2TGTP       | pCNX derivative containing *pbp2TGTP*(E114Q and S398G) under the control of Pcad promoter; Amp^R^ Kan^R^ | This study          |
| pVraT-GFP       | pMAD with *sgfp* 5' fusion to vraT, Amp^R^, Ery^R^                          | This study          |
| pVraS-mCherry   | pMAD with *mCherry* 3' fusion to vraS, Amp^R^, Ery^R^                      | This study          |
| pVraT-PhoB      | pCN51 derivative containing *phoB* 3' fusion to vraT under the control of Pcad promoter, Amp^R^, Ery^R^ | This study          |
| pPhoB_VraT      | pCN51 derivative containing *phoB* 5' fusion to vraT under the control of Pcad promoter, Amp^R^, Ery^R^ | This study          |
### Supplementary Table 2. Strains used in this study.

| Strains | Description | Source or reference |
|---------|-------------|---------------------|
| **Escherichia coli** | | |
| DC10B | $\Delta$dam in the DH10B background; Dam methylation only | 10 |
| **Staphylococcus aureus** | | |
| COL | HA-MRSA | 11 |
| RN4220 | Restriction-deficient derivative of NCTC8325-4 | 12 |
| COL Pvra-sGFP | COL with pFAST3-Pvra; Ery<sup>R</sup> | This study |
| ColPBP1TP | COL tet<sup>3</sup> pbpA::pbpA<sup>5214A</sup> | 13 |
| COLΔpbpB | pbpB deletion in COL | This study |
| COLΔpbp3 | pbp3 deletion in COL | 13 |
| COLΔpbpD | pbpD deletion in COL | 14 |
| COLΔmgt | mgt deletion in COL | 15 |
| COLΔsgtA | sgtA deletion in COL | 15 |
| COLΔmecA | mecA deletion in COL | 16 |
| ColPBP1TP_Pvra-sGFP | COL tet<sup>3</sup> pbpA::pbpA<sup>5214A</sup> with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔpbpB_Pvra-sGFP | COLΔpbpB with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔpbp3_Pvra-sGFP | COLΔpbpC with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔpbpD_Pvra-sGFP | COLΔpbpD with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔmgt_Pvra-sGFP | COLΔmgt with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔsgtA_Pvra-sGFP | COLΔsgtA with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔmecA_Pvra-sGFP | COLΔmecA with pPvra-pFAST3; Ery<sup>R</sup> | This study |
| COLΔpbpB_Pvra-sGFPpCNX | COLΔpbpB_Pvra-sGFP with pCNX; Ery<sup>R</sup>, Kan<sup>R</sup> | This study |
| COLΔpbpB_Pvra-sGFPpPBP2 | COLΔpbpB_Pvra-sGFP with pPBP2; Ery<sup>R</sup>, Kan<sup>R</sup> | This study |
| COLΔpbpB_Pvra-sGFPpPBP2TG<sup>*</sup> | COLΔpbpB_Pvra-sGFP with pPBP2TG; Ery<sup>R</sup>, Kan<sup>R</sup> | This study |
| COLΔpbpB_Pvra-sGFPpPBP2TP<sup>*</sup> | COLΔpbpB_Pvra-sGFP with pCNX-pbp2TP; Ery<sup>R</sup>, Kan<sup>R</sup> | This study |
| COLΔpbpB_Pvra-sGFPpPBP2TG<sup>*</sup>TP<sup>*</sup> | COLΔpbpB_Pvra-sGFP and pPBP2TGTP, Ery<sup>R</sup>, Kan<sup>R</sup> | This study |
| BCBPM073 | COL pbpB::sgfp-pbpB | 17 |
| COLsGFP_VraT | COL vraT::sgfp-vraT | This study |
| COLsGFP-VraTVraS-mCherry | COL vraT::sgfp-vraT, vraS::vraS-mcherry | This study |
| COLΔphoB | phoB deletion in COL | Veiga & Pinho |
| COLΔ\(\text{phoB}\)pCN51     | \(\text{ColΔphoB with pCN51; Ery}^R\) | This study |
|-------------------------------|----------------------------------------|------------|
| COLΔ\(\text{phoB}\)pVraT-\(\text{PhoB}\) | \(\text{ColΔphoB with pVraT}_\text{PhoB}; Ery}^R\) | This study |
| COLΔ\(\text{phoB}\)p\(\text{PhoB-VraT}\) | \(\text{ColΔphoB with pPhoB}_\text{VraT}; Ery}^R\) | This study |
| Primer name | Sequence (5'-3') |
|-------------|-----------------|
| PVraSR_P1_KpnI | gctgcggtaccgcgtgctatgttctgcgc |
| PVraSR_P2_New_Xho | cgcgcgctgatataaataagtttaaatgcccaaatgcc |
| PBP2KO-P1 | acagcaatccaaataactctcgtgc |
| PBP2KO-P2 | tagttgaatatatcgcgtatagcggtctctacttc |
| PBP2KO-P3 | tgaggacccgctgtagggatatatttcaactatc |
| PBP2KO-P4 | acgcaggattctgtccactttagagatgg |
| PBP2muts_P1 | ggtgcactctagaggatccccctccggtgtgatattagata |
| PBP2muts_P2 | aagtgaggcccgggtatagcccgcagaaagatcttcttctt |
| P1pMADpbp2TP | ggcgccccggatcaaatatccttttacttacttc |
| P2pMADpbp2TP | cgcggatcaccagtagggtgagtc |
| P3pMADpbp2TP | cgcggatcccaaaaactttcttagcctcttct |
| P4pMADpbp2TP | cgcgtgcagcacttttaaaagatcttct |
| P1pCNXpbp2 | caagtcgatcggatcttttctctctgatgatagtttct |
| P2pCNXpbp2 | cgcctgaattcgagctcggtaccctcaactctcacttct |
| E114Q fw | gtactcgcgactcaagacaatcgtttctacgaacatg |
| E114Q rev | cgcgtctggattctgtttatagatggacttacttct |
| VraSCterfwdNcoI | gcgcgcggatgggtacaagtggtctctttcacttttc |
| VraSmchMLbwd | agaacccagcagcggcaggagccagaaatatttaaggtgttcttacttt |
| mCherryMLfwd | tccggtcgtcttctgctgactgttagttgaagtaaggtgagttctt |
| mCherrybwd2 | atacgaatctctctcttagtacagcgcgtcacgcagcccagc |
| Vrasfwd3 | aagagagatcgtgtagtacgaatttaagatagtgtggtgagat |
| VraBrdBamHI | gcgcgccgatctctcttagtacagcgcgtcactgtcactg |
| orf1_fw_EcoRI | gcgcggagtgacgagtc gccggtcagcttttcaatccaatctcag |
| orf1_rev_link_sgfp | cttgacagacgcgctcttctacttacttctaagttgat |
| sgfp_fw_link_orf1 | gaccgccgatcggatcttttctctctgatgatagtttctt |
| sgfp_rev_link_vraT | gacatcgcgcgatcggatcttttctctctgatgatagtttctt |
| vraT_fw_link_sgfp | tccggtcgtcgcgtgctgtgctgactgttagttgatagtttctt |
| vraT rev bamHI | gcgcggatcgcgctcttttctctctgatgatagtttctt |
| phoB_vraT_P1 |gtcgcggatcgcgctcttttctctctgatgatagtttctt |
| phoB_vraT_P2 | cagcgcggctcttttctctctgatgatagtttctt |
| phoB_vraT_P3 | caagtcgctggcggcgggccctcatgcacacacaaatataatc |
| phoB_vraT_P4 | cccggtcgtccggttctgctgactgttagttgatagtttctt |
| vraT_phoB_P1 | gttgcgcggatcgcgctcttttctctctgatgatagtttctt |
| vraT_phoB_P2 | ggatcgcggatcgcgctcttttctctctgatgatagtttctt |
| vraT_phoB_P3 | cgcggatcgcgctcttttctctctgatgatagtttctt |
| vraT_phoB_P4 | gcgcggatcgcgctcttttctctctgatgatagtttctt |
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