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A novel mode of regulation of the *Staphylococcus aureus* Vancomycin-resistance-associated response regulator VraR mediated by Stk1 protein phosphorylation

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ABSTRACT

The *Staphylococcus aureus* Vancomycin-resistance-associated response regulator VraR is known as an important response regulator, member of the VraTSR three-component signal transduction system that modulates the expression of the cell wall stress stimulon in response to a number of different cell wall active antibiotics. Given its crucial role in regulating gene expression in response to antibiotic challenges, VraR must be tightly regulated. We report here for the first time in *S. aureus* convergence of two major signal transduction systems, serine/threonine protein kinase and two (three)-component systems. We demonstrate that VraR can be phosphorylated by the staphylococcal Ser/Thr protein kinase Stk1 and that phosphorylation negatively affects its DNA-binding properties. Mass spectrometric analyses and site-directed mutagenesis identified Thr106, Thr119, Thr175 and Thr178 as phosphoacceptors. A *S. aureus* Δ*vraR* mutant expressing a VraR derivative that mimics constitutive phosphorylation, VraR_Asp, still exhibited markedly decreased antibiotic resistance against different cell wall active antibiotics, when compared to the wild-type, suggesting that VraR phosphorylation may represent a novel and presumably more general mechanism of regulation of the two (three)-component systems in staphylococci.

1. Introduction

Signal transducing mechanisms are essential for regulation of gene expression in both prokaryotic and eukaryotic organisms. Regulation of gene expression in eukaryotes is accomplished by serine/threonine and tyrosine kinases and cognate phosphatases. In contrast, gene expression in prokaryotes is often controlled by two-component systems that are comprised of a sensor histidine kinase and a cognate DNA-binding response regulator. Pathogenic bacteria utilize two-component systems in many cases to regulate expression of their virulence factors and for adaptive responses to the external environment. It has been previously shown that the human pathogen *Staphylococcus aureus* encodes a single eukaryotic-type serine/threonine kinase, Stk1. Recent studies provided first insights into the regulatory function of Stk1 and the molecular mechanisms of the Ser/Thr phosphorylation system in *S. aureus* [1]. Interestingly, the Ser/Thr kinase Stk1 was identified to influence central metabolic processes in *S. aureus* [2], and the activity of important regulatory factors such as SarA [3], MgrA [4], LuxS [5], and CcpA [6]. Though, the putative role of Stk1 towards antibiotic adaptation remained to be investigated.

The spread of *S. aureus* strains that are resistant to many of the commonly administered antibiotics is a significant threat to public health throughout the world. There is an urgent need to understand the signalling pathways that allow *S. aureus* to respond to and resist antibiotic chemotherapy treatment and to find new drug targets, in novel biochemical pathways, to combat these infections. In recent years, there has been growing interest in the *S. aureus* Vancomycin-resistance-associated sensor and response regulator (VraTSR) system, a three-component signal transduction system that responds to a number of cell wall active antibiotics by modulating the expression of a set of genes called the "cell wall stress stimulon" [7]. The histidine kinase, VraS, and its cognate response regulator, VraR are part of an operon that includes two further open reading frames, *vraU*, and *vraT* (syn. *yvqF*), with the latter

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gene product, a putative membrane protein, playing a regulatory role in the VraSR-mediated cell wall stress stimulon [8,9]. Mutations or deletions within either *vraT*, *vraS*, or *vraR* affect antibiotic resistance to both β-lactam and glycopeptide antibiotics in a range of *S. aureus* strains including Methicillin resistant *S. aureus* (MRSA) [10,11].

In this work, we demonstrated convergence of the two major signal transduction systems, serine/threonine protein kinase and two (three)-component sytems, for the first time in *S. aureus*. We identified VraR to be phosphorylated and therefore dysregulated by Stk1-mediated phosphorylation. Collectively, our study provides novel evidence that discrete signalling components such as a eukaryotic-type serine/threonine kinase and a two (three)-component response regulator are integrated for co-ordinate regulation.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin or 100 μg/ml spectinomycin when required. *S. aureus* isolates were plated on Tryptic Soy Agar (TSA) supplemented with 10 μg/ml chloramphenicol or 50 μg/ml kanamycin, when required, or grown in filter-sterilized Tryptic Soy Broth (TSB), Müller-Hington (MH) or Brain–Heart Infusion (BHI) mediums at 37 °C and 150 rpm with a culture to flask volume of 1:10.

2.2. Cloning, expression and purification of recombinant VraR and mutant proteins

The *vraR* gene was amplified by PCR using *S. aureus* N315 chromosomal DNA as a template with the primers listed in Table 1 containing an *Nde*I and a *Bam*HI restriction site, respectively. The corresponding amplified product was digested with *Nde*I and *Bam*HI, and ligated into the pETPhos plasmid [12], a variant of pET15b (Novagen) that includes a tobacco etch virus (TEV) protease site instead of the thrombin site, and a N-terminal His-tag free of Ser/Thr/Tyr residues, thus generating pETPhos_*vraR*. pETPhos_*vraR* derivatives harboring threonine to alanine or aspartic acid substitutions were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The duet strategy was used to generate a hyper-phosphorylated VraR protein as described previously [6,13]. The *vraR* gene was cloned into the pCDFDuet-1 vector containing the Stk1 kinase domain [5], generating plasmid pDuet_*vraR*, which was used to transform *E. coli* BL21(DE3)Star cells. pDuet_*vraR E. coli* cells were used to overexpress and purify His-tagged VraR as previously described [6].

2.3. Overexpression of VraR in S. aureus

The *vraR* gene was amplified by PCR using *S. aureus* N315 chromosomal DNA as a template with the primers listed in Table 1 containing a *Bam*HI and a *Pst*I site, respectively. The PCR product was digested with *Bam*HI/*Pst*I, enabling direct cloning into the pMK4-pProt expression vector cut with the same enzymes, thus generating pMK4_*vraR*, which allows constitutive expression in *S. aureus* as previously described [6,14]. The mutants corresponding to Thr to Ala or Asp were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) generating the pMK4_*vraR_Ala* and the pMK4_*vraR_Asp* constructs. The resulting vectors were used to transform *S. aureus* strain RN4220 which served as donor for transducing the plasmids into *S. aureus* BB270 Δ*vraR* [10].

2.4. In vitro kinase assay

In vitro phosphorylation was performed with 4 μg of VraR and 1 μg of Stk1 as described previously [6].

2.5. Electrophoretic mobility shift assays

The DNA probes for electrophoretic mobility shift assays (EMSA) were generated by PCR using *S. aureus* N315 chromosomal DNA as a template which encompass the promoter region of *vraRS operon* as previously described with respective primers pairs listed in Table 1 [15]. The 5′ ends of the double-stranded PCR products were labeled using [γ-^{32}P]-ATP and T4 polynucleotide kinase. A typical assay mixture contained in 20 μl: 10 mM Tris–HCl, pH 7.5; 50 mM KCl; 1 mM dithiothreitol (DTT); 5 mM $MgCl_2$; 0.1 μg of nonspecific competitor (polydI-dC); 2.5% (v/v) glycerol; 0.05% (v/v) Igepal; radioactive DNA probe (2000 cpm ml^{-1}) and various amounts (0, 15, 65, 130, 200 nM) of the purified VraR proteins. After 20 min of incubation at room temperature, 20 μl of this mixture was loaded onto a native 6% polyacrylamide TBE retardation gel (Novex®, Invitrogen) and electrophoresed in 1× TBE (v/v) buffer

Table 1
Primers used in this study.

Primers	5′ to 3′ sequence[a,b]
VraR WT dir pETPhos	TAATAGctCATATGACGATTAAAGTATTGTTTGTGGATGATCATG (NdeI)
VraR WT rev pETPhos	TATGGATCCTTACTATTGAATTAAATTATGTTGGAATGC (BamHI)
VraR WT dir pETDuet	AGGAGATATACCATGGGCGGCCACCATCATCATCAT (NcoI)
VraR WT rev pETDuet	GCTCGAATTCGGATCCCTATTGAATTAAATTATGTTGGAATGC (BamHI)
VraR WT dir pMK4-pProt	GGAGGATCCATGGACTACAAGGACCACGACGGCGACTACAAGGACCACGACATCGACTACAAGGACGATGACGACAAG (BamHI)
VraR WT rev pMK4-pProt	CTGCTGCAGCTATTGAATTAAATTATGTTGGAATGC (PstI)
VraR T106A dir	ATGCAGGTGTCGATAGTTACATTTTAAAA**G**CAACAAGTGCAAAAGA
VraR T106D dir	TGCATTAGATGCAGGTGTCGATAGTTACATTTTAAAA**GAT**ACAAGTGCAAAAGATATCG
VraR T119A dir	GCCGATGCAGTTCGTAAA**G**CTTCTAGAGGAGAATCT
VraR T119D dir	GCAAAAGATATCGCCGATGCAGTTCGTAAA**GAT**TCTAGAGGAGAATCT
VraR T175A dir	GCTAGTGCATCGCATATT**G**CTATTAAAACGGTTAAG
VraR T178A dir	tcgcatATTACTATTAAA**G**CGGTTAAGACACATGTG
VraR T175_178A dir	AAATCAAGAAATTGCTAGTGCATCGCATATT**G**CTATTAAA**G**CGGTTAAGACACATGT
VraR T175_178D dir	GCTAGTGCATCGCATATT**GAT**ATTAAA**GAT**GTTAAGACACATGTGAG
Promoter region of VraSR operon dir	ACGAAGCTTGGTCCGATTTTAACGACAAAAATTG
Promoter region of VraSR operon rev	TGAAATGACGCATTGATTGTGTTC

[a] Restriction sites are underlined and specified into brackets.
[b] Mutagenized bases are shown in bold.

for 1 h at 100 V cm^{-1}. Radioactive species were detected by autoradiography using direct exposure to films.

2.6. Mass spectrometry analysis

Purified His-tagged hyper-phosphorylated VraR from the *E. coli* strain carrying pDuet_*vraR* and co-expressing the Stk1 kinase, was subjected to mass spectrometry without further treatment. Subsequent mass spectrometric analyses were performed as previously reported [6,16].

2.7. Antibody production and immunoblotting

Polyclonal anti-VraR antibodies were raised by injecting 500 μg of the His-tagged recombinant VraR into rats (Eurogentec, Liege, Belgium). The resulting crude antisera were purified against the immobilized VraR antigen. For the determination of VraR expression in *S. aureus*, cytoplasmic protein extracts were isolated from *S. aureus* cell cultures grown for 5 h in TSB at 37 °C as previously described [17], and protein fractions were separated using SDS–PAGE, blotted onto a nitrocellulose membrane, and subjected to Western blot analysis using the antigen-purified polyclonal anti-VraR antiserum and revealed with secondary antibodies labeled with IRDye 800CW infrared dyes (LiCOR). Phosphorylated VraR isoforms were separated by SDS–polyacrylamide gel containing $MnCl_2$ and Phos-tag acrylamide (Wäko Chemicals, Japan) and subjected to Western blot analysis as described above.

2.8. Antibiotic resistance tests

MICs of antibiotics were determined by Etest (Biomérieux) according to the manufacturer's instructions. Broth microdilutions were performed according to CLSI M07-A9 guidelines. Plates for resistance tests were supplemented with chloramphenicol (10 μg/ml) to ensure plasmid maintenance.

3. Results and discussion

3.1. Stk1-mediated phosphorylation of VraR

The *S. aureus* genome encodes one Ser/Thr protein kinase named Stk1 or PknB [2,14]. While Stk1 appears to be involved in different key pathways like cell wall metabolism, antibiotic susceptibility, central metabolism, and regulation of virulence [1–3,6,18–20], only little is known about the nature of the corresponding substrates. Due to our interests in Ser/Thr kinase regulation in *S. aureus*, and in antibiotic resistance pathways of this pathogen, we were interested in investigating whether the response regulator (VraR), might be regulated by Ser/Thr phosphorylation, as it is already known to be regulated by the histidine kinase VraS in this organism.

In order to establish whether the VraR protein could undergo Stk1-mediated phosphorylation, we first investigated *in vitro*, whether VraR could be phosphorylated in the presence of the purified Stk1 kinase. Stk1 was expressed as a His-tagged fusion protein and purified from *E. coli* as described previously [3,5]. The purified kinase was incubated with VraR and [γ-^{33}P]-ATP, the proteins resolved by SDS–PAGE, and the phosphorylation profile analyzed by autoradiography (Fig. 1A). The upper bands illustrate the autokinase activity of Stk1, while the lower bands represent phosphorylated VraR proteins (VraR-P). The presence of an intense radioactive signal indicated that VraR was indeed phosphorylated by Stk1. As expected, no radioactive bands were observed in the absence of Stk1 in this assay. These data indicated that VraR is phosphorylated by Stk1, and suggested that this protein is regulated via this Ser/Thr kinase.

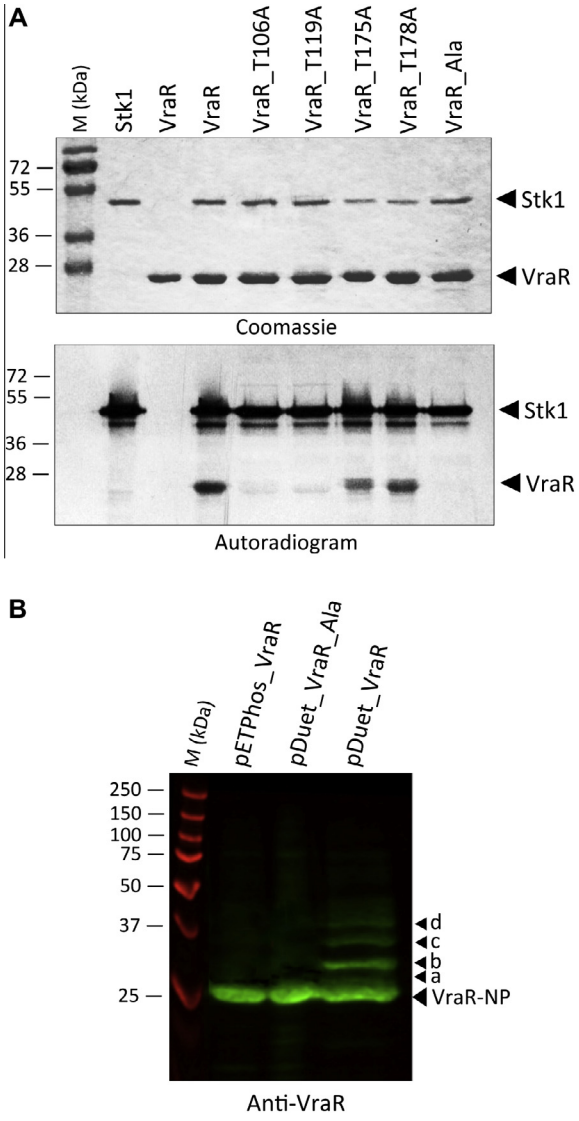


Fig. 1. (A) *In vitro* phosphorylation of VraR and mutant derivatives. The recombinant Stk1 kinase encoded by the *S. aureus* genome was expressed and purified as a His-tagged fusion in *E. coli* and incubated in presence of [γ-^{33}P]-ATP with either the purified His-tagged *S. aureus* VraR, or the mutated variants VraR_T106A (harboring a Thr to Ala substitution at T106), VraR_T119A (harboring a Thr to Ala substitution at T119), VraR_T175A (harboring a Thr to Ala substitution at T175), VraR_T178A (harboring a Thr to Ala substitution at T178), or VraR_Ala (harboring Thr to Ala substitutions at T106, T119, T175, T178). Samples were separated by SDS–PAGE, stained with Coomassie Blue (upper panel), and visualized by autoradiography (lower panel). The upper bands illustrate the autokinase activity of Stk1, and the lower bands represent phosphorylated VraR proteins. Standard proteins of known molecular masses were run in parallel (kDa lane). (B) Phosphorylation status of VraR in a Stk1 co-expressing *E. coli* strain. The *vraR* and *vraR_Ala* alleles were cloned into pETDuet_*stk1*. Four micrograms of the purified His-tagged VraR variants were run on Phos-tag SDS–PAGE gel, probed with anti-VraR antibodies and revealed with secondary antibodies labeled with IRDye 800CW infrared dyes (LiCOR). VraR-NP corresponds to unphosphorylated VraR. a, b, c, d highlight phosphorylated state of VraR.

3.2. VraR is phosphorylated on four threonine residues

Mass spectrometry (MS) was subsequently used to identify the number and nature of phosphorylation sites on VraR, as reported previously for other STPK substrates [5,6,16,21]. Phosphorylated VraR-P was purified from *E. coli* co-expressing Stk1 and VraR (pDuet_*vraR*) and subjected to mass spectrometry analysis after tryptic digestion. We obtained a sequence coverage of 97%, bearing all possible Ser and Thr residues. The ProteinPilot® database searching

software, using the Paragon method with phosphorylation emphasis, was then used to detect and identify the phosphorylated peptides. The MS/MS spectra unambiguously identified the presence of four phosphate groups (Table 2), thus indicating that VraR is phosphorylated on four threonines, corresponding to Thr106, Thr119, Thr175 and Thr178.

To confirm the phosphorylation sites identified by MS/MS, we substituted the phosphosites by alanines, either individually or together by site-directed mutagenesis to introduce either single or quadruple mutations (Thr to Ala) in VraR. The corresponding VraR derivatives were expressed and purified as His-tagged proteins in *E. coli* BL21(DE3)Star cells. Following incubation with Stk1 and [γ-^{33}P]-ATP, SDS–PAGE/autoradiogram revealed that phosphorylation was partially inhibited in the VraR_T175A and VraR_T178A mutants and this inhibition was even more pronounced in the VraR_T106A and VraR_T119A mutants, which seem to indicate their critical role in VraR phosphorylation (Fig. 1A). Complete abrogation of the phosphorylation reaction was evidenced in the quadruple VraR_T106A/T119A/T175A/T178A (VraR_Ala) phosphoablative mutant, thus confirming the proper identification of the sites (Fig. 1A).

To further address the relevance of *in vitro* phosphorylation, the phosphoablative *vraR_Ala* allele was cloned into the pCDFDuet-1 vector [5], which allows its co-expression together with Stk1 in *E. coli*. The purified VraR_Ala was next assessed for phosphorylation in an *in vivo* context. Therefore, in order to track the level of phosphorylated VraR and confirm that the phosphoablative mutant was not phosphorylated anymore *in vivo*, we took advantage of the Phos-tag compound, which selectively forms a complex with phosphorylated amino acids in the presence of divalent cations [22]. In the presence of $MnCl_2$, Phos-tag induces a mobility shift in phosphorylated proteins in SDS–PAGE, causing them to migrate slower than the non-phosphorylated form. This method, originally developed for the study of eukaryotic Ser/Thr kinases, has been successfully adapted for bacterial histidine kinases (e.g. *E. coli* PhoP), and validated against standard methods that require radioactive ^{32}P-labelling [23]. We used Phos-tag SDS–PAGE followed by Western immunoblotting with purified VraR antiserum to simultaneously detect the phosphorylated and the unphosphorylated forms of VraR. First, the specificity of the Phos-tag for the phosphorylated VraR isoform was demonstrated using the protein purified from *E. coli* co-expressing Stk1 together with VraR wild-type (Fig. 1B) corresponding to pETDuet_VraR. As anticipated, pETDuet_VraR displayed four different slowly migrating phospho-VraR bands (labeled a, b, c and d in Fig. 1B) among remaining non-phosphorylated VraR (VraR-NP). We deduced that the four phospho-VraR signals correspond to VraR phosphorylated either at one site towards the four identified phosphorylation sites, thus confirming our results about four phosphosites. Our observations are consistent with previous studies, which also reported that Phos-tag binding shifts the mobility of phosphorylated proteins specific to different phosphorylation sites [23]. Phosphobands were absent in the purified non-phosphorylable pETDuet_VraR_Ala protein as well as in the purified protein expressed without its specific Stk1 kinase (pETPhos_VraR) (Fig. 1B), thus confirming that VraR is specifically phosphorylated by Stk1 in this *in vivo* system.

3.3. Localization of the phosphoacceptors on the VraR structure

To get more insights into the possible role of Stk1-mediated phosphorylation on VraR, we looked at the crystal structure of VraR. Three out of the four Stk1-mediated phosphorylation sites are located in key residues for the VraR activity and regulation. The residues T175 and T178 are located on the Helix-Turn-Helix (HTH) binding domain of VraR. More precisely, they are located at the beginning and in the middle of the α-helix α9, respectively (residues I176 to L190). The reported structures of VraR in its inactive monomer and active dimer forms have recently been published [24]. Then, structural analysis of the activated VraR demonstrated the role of this α-helix α9 for the DNA-binding recognition, thus being in accordance with the previous hypothesis [25]. In the dimer, the two α9 helices are positioned to create a large electropositive DNA-binding surface. Therefore, the incorporation of negative charges within the α9 helix will impede DNA-binding (Fig. 2A). Two other phosphorylation sites are located at the other side of the protein, in the VraR regulator domain. Upon activation, VraR is phosphorylated on the Asp55 and leads to dimerization which is (i) promoted by structural rearrangement as described by Leonard et al. (2013) [24]; and (ii) mediated by hydrophobic contact between two monomers around the α-helix α1 (residues E12 to S23). A key residue for this interaction is the methionine M13 docked into a hydrophobic pocket within the dimerization interface comprises of the α-helix α1 and the α-helix α5 (residues A109 to S120). If the residue M13 is not in contact with the threonine T106 (distance Cα–Cα is 8.7A), as might be the case upon phosphorylation of the T106, it will disturb the hydrophobic request for the protein dimerization, thus preventing VraR activation (Fig. 2B).

3.4. Phosphorylation negatively regulates VraR DNA-binding properties

The structural mapping of the phosphorylation sites suggested that the phosphorylation of VraR might negatively influence the DNA-binding activity of this protein. To test this hypothesis, we took advantage that VraR was already known to be able to bind to its own promoter preceding *vraS*p [15]. Therefore, we analyzed the binding capacities of the non-phosphorylated VraR form (VraR) and that of the hyper-phosphorylated VraR form (VraR-P) (Fig. 3). While the unphosphorylated VraR interacted with the double-stranded DNA probe *vraS*p tested (Fig. 3A), the phosphorylated VraR-P failed to shift any of these probes (Fig. 3B), indicating that VraR-P had lost its ability to bind to its *vraS* promoter sequence. However, our EMSA carried out with the VraR_Ala phosphoablative mutant protein showed that this VraR derivative was also impaired in its DNA-binding activity (Fig. 3C), thus highlighting the critical

Table 2
Phosphoacceptors identified after purification of *S. aureus* VraR from the *E. coli BL21(DE3)star* strain co-expressing *S. aureus* Stk1. Sequences of the phosphorylated peptides identified in VraR as determined by mass spectrometry following tryptic digestion are indicated, and phosphorylated residues (pT) are shown in bold.

Phosphorylated tryptic peptide sequence of VraR purified from pCDFDuet co-expressing Stk1	Number of detected phosphate groups LC/MS/MS	Phosphorylated residue(s)
[94–110] ALDAGVDSYILK**pT**TSAK	1	T106
[94–117] ALDAGVDSYILK**pT**TSAKDIADAVR	1	T106
[118–133] K**pT**SRGESVFEPEVLVK	1	T119
[110–133] DIADAVRK**pT**SRGESVFEPEVLVK	1	T119
[105–133] TTSAKDIADAVRK**pT**SRGESVFEPEVLVK	1	T119
[162–177] GYSNQEIASASHI**pT**IK	1	T175
[162–180] GYSNQEIASASHITIK**pT**VK	1	T178

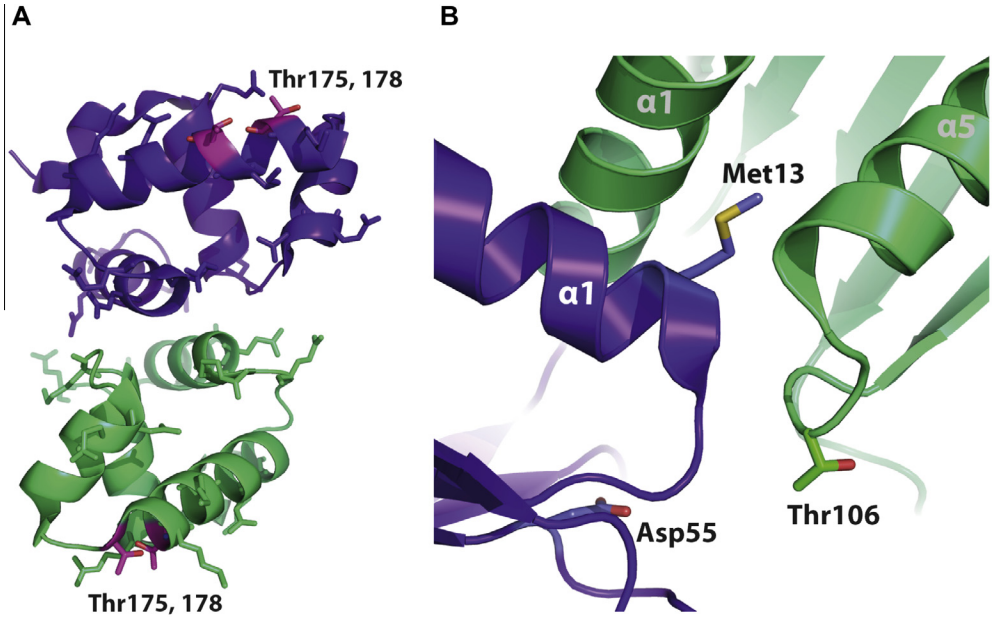


Fig. 2. Mapping of the VraR phosphorylation sites. The structure of the activated form of VraR (PDB4IF4) is represented as a cartoon and each monomer are colored either in green or purple. (A) Two phosphorylation sites (T175 and T178) are located on the DNA-binding domain. The phosphorylation will bring electronegative charge on a positively charged surface (residues KRQN are represented with sticks). (B) Two phosphorylation sites are located on the regulator domain (T106 and T119). The phosphorylation of T106 will bring a negative charge in an hydrophobic environment and could impaired the dimerization leads by hydrophobic contact, noteworthy by the methionine residue M13. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

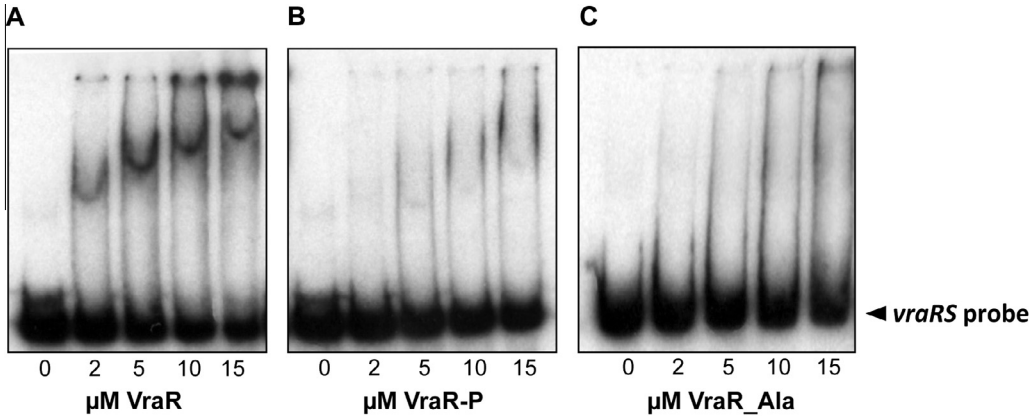


Fig. 3. DNA-binding activity of VraR derivatives. Gel electrophoretic mobility shift assays (EMSA) of VraR binding to the *vraRS* promoter. The promoter region of the *vraRS* operon was amplified by PCR, radioactively labeled, and incubated with 2, 5, 10 and 15 μM of purified VraR. (A) Binding of the unphosphorylated VraR (VraR), (B) phosphorylated VraR (VraR-P), or (C) VraR_Ala mutant to the *vraRS* promoter region.

role of phosphorylation but also of the natures of the residues on the DNA-binding capacity of this regulatory protein. Noteworthy, these effects were not due to a tertiary structure change, as confirmed by analysis of the trypsinolysis kinetics of wild type and mutated VraR proteins (Fig. S1). Thus, it can be inferred that VraR phosphorylation in *S. aureus* reduces/abolishes its activity, supporting the view that this post-translational modification plays a critical role in regulating VraR activity.

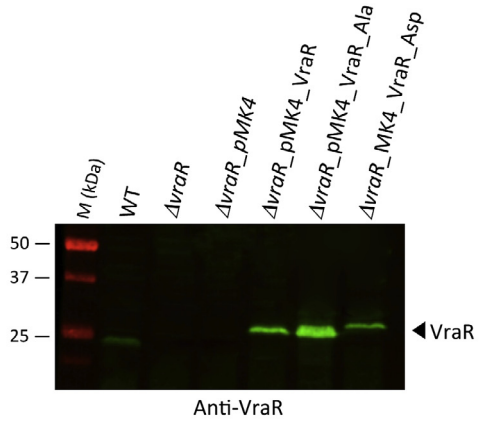
Taken together, these results indicate that Stk1-phosphorylation negatively affects the DNA-binding properties of VraR rather than altering the overall structure of the protein.

3.5. Lack of antibiotic resistance complementation with the VraR phosphomimetic mutant in a ΔvraR mutant strain

In order to test the role of Stk1-mediated phosphorylation in *S. aureus* regarding antibiotic resistance, we assessed the role of the specific phosphosites identified. Acidic residues such as aspartic acid (Asp) qualitatively mimic the phosphorylation effect with regard to functional activity [16,21,26]. Therefore, phosphoablative (Thr to Ala replacements) and phosphomimetic (Thr to Asp replacements) *VraR* alleles were generated and cloned into the shuttle vector pMK4-pProt, and the resulting plasmids, pMK4_*vraR*, pMK4_*vraR_Ala* and pMK4_*vraR_Asp*, respectively, were transformed into the *S. aureus* strain BB270 Δ*vraR* [10]. In these strains, wild-type, phosphoablative and phosphomimetic VraR isoforms expression was monitored by Western blotting using anti-VraR antibodies. As expected, *S. aureus* BB270 Δ*vraR* failed to express VraR, while increased amounts of VraR were synthesized in the BB270 Δ*vraR* derivatives complemented with the phosphoablative *vraR* variant (pMK4_*vraR_Ala*), the phosphomimetic (pMK4_*vraR_Asp*) and the *vraR* wild-type allele (pMK4_*vraR*), respectively (Fig. 4A), probably due to a multi-copy effect induced by transformation with the pMK4 derivatives.

The impact of constitutive VraR phosphorylation on specific antibiotic resistance known to be associated with this regulator

A



B

Strains	MICs		
	Vancomycin	teicoplanin	bacitracine
S. aureus BB 270 wt	2	1,5	96
S. aureus BB 270 Δ *vraR*	1	0,064	24
S. aureus BB 270 Δ *vraR*/pMK4 empty	1	0,064	16
S. aureus BB 270 Δ vraR/pMK4 VraR	2	1	48
S. aureus BB 270 Δ vraR/pMK4 VraR_Ala	0,75	0,047	16
S. aureus BB 270 Δ vraR/pMK4 VraR_Asp	1	0,0064	24

Fig. 4. Complementation of the *vraR* antibiotic resistance phenotype in *S. aureus*. (A) Western blot analysis of cytosolic protein extracts obtained from cultures of *S. aureus* strain BB270, its respective Δ*vraR* mutant, and the Δ*vraR* derivatives complemented with the wild type allele (pMK4_*vraR*), the VraR_Ala variant (pMK4_*vraR_Ala*), and the VraR_Asp variant (pMK4_*vraR_Asp*), with the polyclonal anti-VraR antibody. (B) MICs were performed on *S. aureus* BB270 and derivative mutant strains.

was next examined. In line with previous findings [10], we found an almost complete complementation of the antibiotic resistance phenotype in the Δ*vraR* mutant trans-complemented with *vraR* wild-type allele, while VraR_Ala or VraR_Asp expressing strains were unable to complement the resistance profile (Fig. 4B), although introduction of the VraR_Ala or VraR_Asp versions did neither alter the expression of both proteins nor their folding (Figs. 4A and S1). This result suggests that the threonine residues involved in this regulation are critical for VraR activity *in vivo*, presumably as a consequence of its impaired DNA-binding activity towards specific regulated genes involved in these antibiotic resistance processes.

In conclusion, we provide here the first evidence of a Ser/Thr kinase mediated modification involved in the regulation of VraR activity in *S. aureus*. Our results strongly suggest that phosphorylation of VraR may play a role in regulating the levels of antibiotic resistance in addition to the previously shown regulation via VraS and VraT. Thus, it appears that *S. aureus* may control in a very subtle manner the equilibrium between STPK-mediated phosphorylation compared to classical two (three)-component activation. Whether Stk1 driven phosphorylation occurs under certain circumstances such as antibiotic stress in regulating the major regulator VraR requires further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.128.

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