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Two Unique Phosphorylation-Driven Signaling Pathways Crosstalk in *Staphylococcus aureus* to Modulate the Cell-Wall Charge: Stk1/Stp1 Meets GraSR

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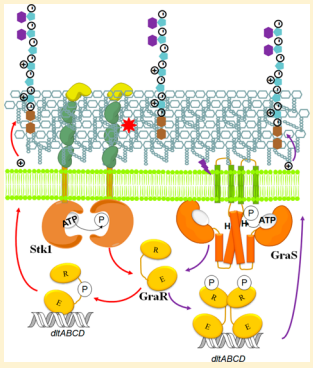
Supporting Information

ABSTRACT: The Stk1/Stp1 and GraSR signal-transduction pathways are two distinct pathways in *Staphylococcus aureus* that rely on a reversible phosphorylation process in transducing external stimuli intracellularly. Stk1/Stp1 is an eukaryote-like Ser/Thr kinase phosphatase pair involved in purine biosynthesis, cell-wall metabolism, and autolysis. GraSR is a two-component system involved in resistance to cationic antimicrobial peptides. Both systems are implicated in *S. aureus* virulence and resistance to cell-wall inhibitors. Our study shows that the response regulator protein GraR undergoes phosphorylation by Stk1 at three threonine residues in the DNA-binding domain. Phosphorylation by Stk1 depends on the structural integrity of GraR as well as the amino acid sequences flanking the phosphorylation sites. Its homologue in *Bacillus subtilis*, BceR, which harbors two of the three phosphorylation sites in GraR, does not undergo Stk1-dependent phosphorylation. GraR is involved in regulation of the *dltABCD* operon, the gene products of which add the D-Ala on wall teichoic acid (WTA). Investigation of WTA isolated from the *S. aureus* RN6390 Δ*graR* strain by NMR spectroscopy showed a clear negative effect that *graR* deletion has on the D-Ala content of WTA. Moreover, complementation of Δ*graR* mutant with *graR* lacking the Stk1 phosphorylation sites mirrors this effect. These findings provide evidence that GraR is a target of Stk1 in vivo and suggest that modification of WTA by D-Ala is modulated by Stk1. The crosstalk between these two otherwise independent signaling pathways may facilitate *S. aureus* interaction with its environment to modulate processes such as cell growth and division and virulence.



Cells sense and translate environmental cues through phosphorylation-mediated signal-transduction pathways (protein networks that are intraconnected through reversible phosphorylation processes). In eukaryotes, signaling relies on modulation of protein activity through phosphorylation at specific serine, threonine, or tyrosine residues by kinases. This process is coupled to dephosphorylation reactions catalyzed by phosphatases. These signaling pathways are essential in cell cycle control and differentiation.[1,2] In prokaryotes, signaling is primarily conducted by two-component signal-transduction systems (TCSs) consisting of a histidine kinase (HK) receptor and its cognate response regulator (RR) protein.[3] However, several studies have shown that eukaryote-like serine/threonine signaling systems are present in prokaryotes[4] and are involved in stress response,[5] biofilm formation,[6] cell-wall biosynthesis,[7−9] cell division,[8] virulence,[10−12] sporulation,[6,13] and antibiotic resistance.[9,12]

Eukaryote-like serine/threonine signaling systems comprise two proteins, a serine/threonine kinase (STK) and a phosphatase (STP). STKs are membrane-bound proteins with an N-terminal domain located in the cytosol and a C-terminal domain protruding outside of the cell (Figure 1). The kinase activity is associated with the N-terminal domain, and the C-terminal domain harbors one to five penicillin-binding protein and serine/threonine kinase-associated domains (PASTA) that are suggested to interact with the cell wall.[14,15] The STPs are soluble cytoplasmic proteins.[14]

Staphylococcus aureus has one major STK and one major STP, referred to herein as Stk1 and Stp1, respectively. Stk1 has also been referred to as PknB or PkrC. A second hypothetical cytoplasmic STK has also been identified in other *S. aureus* strains such as MRSA252, Mu50, N315, JH1, and JH9 and is located in the *SCCmecc* element.[14] *Stk1* and *stp1* are cotranscribed and translationally coupled.[9,10] Stk1 was first identified as a methicillin-resistance factor in a methicillin-resistant *S. aureus* strain.[16] Transcriptomic studies show that *stk1* has a strong regulatory effect on purine biosynthesis, cell-wall metabolism, autolysis, and glutamine synthesis.[11] Stk1 has also been implicated in regulation of virulence.[17,18] Deletions of both *stk1* and *stp1* introduce major defects in cell division and cell-wall morphology and result in an increase of *S. aureus* sensitivity to cephalosporin and carbapenems.[9] Deletion of *stp1* alone results in cell-wall thickening,[9] increased susceptibility to vancomycin, and decreased virulence.[12]

TCS GraSR is involved in *S. aureus* resistance to cationic antimicrobial peptides (CAMPs)[19] and has also been implicated in

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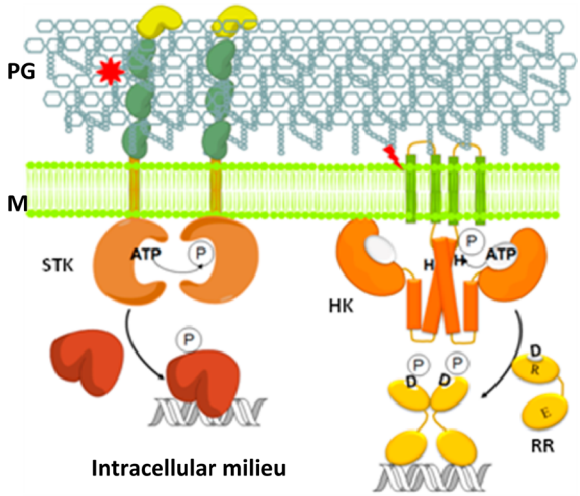

Figure 1. Schematic representation of the signal-transduction pathways mediated by STKs and TCSs. PG indicates peptidoglycan and M indicates plasma membrane. In Stk1, the extracellular portion of the protein is composed of 3 PASTA domains (green shapes) and an immunoglobuline-like domain (yellow shape) and the cytoplasmic domain consists of the kinase domain (brown shape). Targets of STKs include transcription factors (dark-brown shape). In a TCS, the membrane-bound HK (orange shape) is composed of the ATP-binding domain and a histidine box that harbors the phosphorylation site, histidine (H). The RR (yellow shape) consists of the receiver domain (R) and the effector domain (E). The red shapes denote extracellular stimuli.

vancomycin resistance.[20] Recently, GraSR was linked to virulence and cell-wall metabolism.[19] GraSR is composed of a membrane-bound HK, GraS, and its cognate RR, GraR, a cytosolic protein (Figure 1). GraR is a transcription factor that belongs to the OmpR family of response regulators. Typically, in a TCS, the transcription-factor activity is modulated by the kinase and phosphatase activities of the cognate HK.[21,22] A typical RR is composed of a conserved N-terminal domain (receiver domain) that houses the phosphorylation site, an aspartate residue, and a nonconserved C-terminal domain that harbors a helix-turn-helix DNA-binding motif (effector domain).[21]

The CAMP response mechanism mediated by GraSR depends on the activity of an ABC transporter, encoded by the *vraFG* operon.[20] This operon is under the direct regulation of GraR. Notably, GraR does not regulate its own promoter (*graXSR*),[23] and expression levels of GraR are not altered by cell-wall-active antibiotics.[19] In vivo studies have suggested that activation of GraR depends on the kinase and phosphatase activities of GraS.[23] The *S. aureus* response to CAMPs consists of an increase in the positive charge of the cell wall. This is achieved by modification of the poly-D-ribitol-phosphate polymers attached to the peptidoglycan of *S. aureus*, referred to as wall teichoic acid (WTA), with D-Ala. The *dltABCD* operon is the sole system dedicated to the modification of WTA by D-Ala and is under the regulation of GraR.[24,25]

Although both the GraSR and Stk1/Stp1 regulatory pathways rely on reversible phosphorylation reactions for activation, these two signaling pathways are considered to act independently of each other. Recent studies have indicated that both systems are involved in the regulation of cell-wall metabolism, virulence, and resistance to vancomycin[12] and that their regulons overlap.[11,19] In particular, it is striking that deletion of *stk1* results in downregulation of the *vraFG* operon,[11] which is described to be under direct regulation by GraSR.[19] In addition, the expression level of *atl*, the gene that encodes the major autolysin in *S. aureus*, AtlA, is downregulated in *stk1* and *graSR* mutants.[11,19]

Herein, we report our study that examines the role of Stk1 in modulating the activity of GraR through phosphorylation. We discovered that GraR is a target of the Stk1 kinase. Phosphorylation of GraR by Stk1 depends entirely on its intact tertiary structure. In addition, phosphorylation of GraR occurs at the N-terminus of its DNA-binding domain and results in increased DNA-binding activity. Moreover, we have discovered a link between Stk1 phosphorylation of GraR and modification of cell-wall charge. Our results suggest the existence of "regulatory hubs" that enable bacteria to control and alter their resources in response to cell needs.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Chemicals and growth media were purchased from Sigma (Oakville, Canada) or Thermo-Fisher (Whitby, Canada). Chromatography media and columns were purchased from GE Healthcare (Quebec, Canada). The *Escherichia coli* strains Nova Blue and BL21(DE3) as well as the cloning and expression plasmids were purchased from EMD4 Biosciences (New Jersey, USA) or GE Healthcare. Restriction enzymes were obtained from New England Biolabs Canada (Pickering, Canada) or Thermo-Fisher. All primers were purchased from Sigma (Oakville, Canada). [γ-^{32}P]ATP was purchased from PerkinElmer LAS Canada Inc. (Toronto, Canada). The ProteoExtract All-in-One Trypsin Digestion Kit was obtained from EMD4 Biosciences. The genome of the *S. aureus* strain Mu50 was obtained from Cedarlane (Burlington, Canada).

Cloning, Expression, and Purification of Stk1 and Stp1. The *stk1* (*SAV*1220 in Mu50, 1995 bp) and *stp1* (*SAV*1219 in Mu50, 744 bp) genes were amplified from the *S. aureus* Mu50 genome (Table 1). The *stp1* amplicon was ligated into the NdeI/EcoRI sites of pET26b. The stk1 amplicon was ligated into pET151/D-TOPO. We used BL21(DE2) as an expression host for both genes. The above cloning strategies resulted in the introduction of no tag and no extraneous amino acids to the N- or C-terminus of Stp1, but it did introduction a hexa-histidine tag at the N-terminus for Stk1 (herein referred to as Stk1). Correct cloning of the genes into the expression vectors was confirmed by DNA sequencing (York University, core facility).

To induce the target proteins, cell cultures grown to the exponential phase were cooled to room temperature, and IPTG was added at 0.5 or 1 mM for Stp1 and Stk1, respectively. The cell cultures were shaken for 18 h at 25 °C for Stp1 or 10 °C for Stk1. Cells were harvested by centrifugation at 7459*g*.

To isolate Stp1, the cell pellet was resuspended in 20 mM of Tris buffer, pH 7.0, supplemented with 5 mM $MgCl_2$. Sonication was used to lyse the cells, and centrifugation at 18 138*g* for 60 min was performed to remove cell debris. The resulting supernatant was loaded onto a DEAE-Sepharose Fast Flow column pre-equilibrated in the loading buffer (20 mM Tris buffer, pH 7.0, supplemented with 5 mM $MgCl_2$). Elution was performed at 4 mL/min with a linear gradient of 500 mM Tris buffer, pH 7.0, supplemented with 5 mM $MgCl_2$. Fractions containing the protein were pooled and concentrated using an Ultracel-10K centrifugal filter (Thermo-Fisher).

To isolate Stk1, the cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.5) supplemented with 300 mM

NaCl, 10 mM imidazole, and Triton-X 100 (0.5% final). The cell lysate, prepared as described above, was loaded onto a 4 mL Ni-NTA column. Elution was performed at 3 mL/min with a step gradient of 10, 25, 50, and 100% of the elution buffer (50 mM sodium phosphate, 300 mM NaCl, and 300 mM imidazole, pH 7.5). Protein-containing fractions were concentrated using an Ultracel-30K centrifugal filter.

All of the purification steps were performed at 4 °C. The homogeneity of the isolated protein was determined by 12.5% SDS-PAGE. The identity of each protein was confirmed by excising the respective SDS-PAGE band, digesting it with trypsin, and analyzing the peptides by mass spectrometry (Advanced Protein Technology Centre, Hospital for Sick Kids, Toronto, Canada).

Cloning, Expression, and Purification of GraR, GraR^C, and GraR^N. The full-length *graR* gene and its effector domain (*graR*^{C}) were amplified from *S. aureus* Mu50. The effector domain spans amino acids 123−224. The cloning of the receiver domain was carried out by introducing a stop codon after residue 134. The primers for GraR and GraR^C harbor the restriction sites for NdeI and HindIII to enable cloning into the pET26b vector (Table 1). The *graR* (or *graR*^{C}) amplicon was ligated into the pSTBlue-1 vector, which was subsequently introduced into NovaBlue. LB agar plates supplemented with 100 μg/mL of ampicillin and 20 μg/mL of X-gal were used for blue and white screening to identify the insertion of the genes. Positive colonies were sequenced for the presence of the insert. The pSTBlue::*graR*(*graR*^{C}) constructs were digested with the NdeI and HindIII restriction enzymes. The released inserts were gel-purified and ligated into the pET26b vector previously digested with the same enzymes. The pET26b::*graR*(*or graR*^{C}) construct was introduced into NovaBlue and ultimately into BL21(DE3). The sequences and proper insertions of the *graR* and *graR*^{C} genes were confirmed by sequencing. This cloning strategy resulted in the introduction of no tag and no extraneous amino acids to either the N- or C-termini of GraR or GraR^C.

To express the N-terminal region of GraR spanning the amino acids 1−134, we introduced a stop codon after residue 134 via site-directed mutagenesis in the pET26b::*graR* plasmid (Stratagene). Expression of the GraR proteins was performed similarly. Briefly, the BL21(DE3) strain harboring one of the pET26b vectors was inoculated into 5 mL of LB broth supplemented with 50 μg/mL of kanamycin and allowed to grow overnight by shaking at 37 °C. The seed culture was diluted 200-fold into fresh TB broth supplemented with 50 μg/mL of kanamycin and gently shaken at 37 °C to an optical density at 600 nm of approximately 0.6. The cell culture was cooled, and protein expression was initiated by the addition of 0.5 mM IPTG. The cells were gently shaken at 18 °C for 16 h.

To isolate the protein, cells were harvested by centrifugation at 7459*g* for 20 min and resuspended in 20 mM Tris buffer, pH 7.5, supplemented with 5 mM $MgCl_2$. The cell content was liberated by sonication, and cell debris was removed by centrifugation at 18 138*g* for 1 h at 4 °C. The cell lysate was resolved by 12.5% SDS-PAGE to assess the protein-expression level. The putative protein band was excised from the gel and subjected to trypsin digestion.

Purification of all three GraR proteins was performed in two steps. The cell lysate was loaded onto a DEAE Sepharose column equilibrated with 20 mM Tris, pH 7.5, with 5 mM $MgCl_2$. Protein elution was performed in a step gradient from 20 to 500 mM Tris, pH 7.5, with 5 mM $MgCl_2$, using a flow rate of 2.5 mL/min. The fractions containing the desired protein were pooled and concentrated. The concentrated sample was loaded onto a Sephacryl S-200 HiPrep 26−60 size-exclusion column (GE Healthcare) equilibrated with 50 mM Tris buffer (pH 7.4) supplemented with 100 mM KCl and 5 mM $MgCl_2$. Fractions containing the pure protein, as assessed by a Coomassie-stained SDS-PAGE gel, were pooled and concentrated. The protein concentration was determined by Bradford assay. The identity of each protein was confirmed by excising the respective SDS-PAGE band, digesting the band with trypsin, and analyzing the tryptic peptides by mass spectrometry/MASCOT (Advanced Protein Technology Centre, Hospital for Sick Kids, Toronto, Canada).

Constructions of the GraR-Thr128Ala/Thr130Ala/Thr149Ala (GraR triple mutant) and the GraR-Thr130Ala (Stk1 single mutant) mutants were carried out using site-directed mutagenesis (Stratagene). The mutagenic primers are provided in Table S1.

Autophosphorylation Activity of Stk1. Stk1 (2 μM) was incubated with 20 μM [γ-^{32}P]-ATP (10 Ci/mmol) in phosphorylation buffer (PB) (25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 10 mM $MgCl_2$) at room temperature. The reactions were quenched at various time intervals by the addition of 5 μL of 5× SDS sample buffer. Quenched reactions were separated by 12.5% SDS-PAGE. The phosphorylation rate constant was determined from three independent experiments and by fitting the data into the equation $I = C(1 - e^{-kt})$, where I is the band intensity measured in pixels by NIH ImageJ (version 1.45s), k is the rate constant, t is time, and C is the proportionality constant between the intensity and concentration of Stk1-P. Erithacus GraFit software (version 5.0.10) was used to fit the data. The effect of Mg and Mn ions on the autophosphorylation of Stk1 was probed as follows: Stk1 (5 μM) was incubated with 20 μM [γ-^{32}P]-ATP (10 Ci/mmol) in 25 mM Tris-HCl, pH 7.5, 1 mM DTT and varying concentrations of either $MgCl_2$ or $MnCl_2$ (2.5 to 20 mM) for 30 min at room temperature. The reactions were quenched with 5× SDS-PAGE sample buffer and separated by 12.5% SDS-PAGE.

Dephosphorylation of Stk1 by Stp1 was attained by following the above protocol for autophosphorylation of Stk1 and adding Stp1 (20 μM) as the last step. The reaction was quenched after a 30 min incubation and resolved by 12.5% SDS-PAGE.

Kinase Activity of Stk1. Stk1 (5 μM) was incubated with the response regulators GraR (10 μM), BceR (10 μM), and VraR (20 μM) with 20 μM [γ-^{32}P]-ATP (10 Ci/mmol) in PB at room temperature for 30 min. Time-dependent phosphorylation of GraR was performed by incubating GraR (25 μM) with Stk1 (10 μM) and 20 μM [γ-^{32}P]-ATP (10 Ci/mmol) in PB. The reaction was quenched at various time intervals by adding 5 μL of the 5× SDS-PAGE sample buffer and resolved by 15% SDS-PAGE. The phosphorylation rate constant was determined from three independent experiments, and data fitting was performed as described for Stk1 phosphorylation.

To investigate the specificity of GraR phosphorylation by Stk1, GraR (20 μM) was boiled for 10 min and then incubated with Stk1 (5 μM) and 20 μM [γ-^{32}P]-ATP in PB for 30 min at room temperature. The reactions were loaded onto 15% SDS-PAGE gels. All of the radioactive SDS-PAGE gels were exposed to an autoradiography cassette, which was scanned using TYPHOON Trio$^+$ (GE Healthcare) and stained using Coomassie blue.

Table 1. Nucleic Sequences of the Primers Used in This Study[a]

name	sequence (5′−3′)
Stp1Dir	GGAATTC*CATATG*CTAGAGGCACAATTTTTTAC
Stp1Rev	AAG*GAATTC*CTCATCATCATACTTTATCACCTTCAATAG
Stk1Dir	CACCATGATAGGTAAAATAATAAATGAAC
Stk1Rev	TTATTAAATATCATCATAGCTGACTTC
Stk1T128/T130Dir	GAAAAACGTGCATTGGCTTGGCAAGATGC
Stk1T128/T130Rev	GCATCTTGCCAAGCCAATGCACGTTTTTC
Stk1T130ADir	GAAAAACGTACATTGGCTTGGCAAGATGC
Stk1T130ARev	GCATCTTGCCAAGCCAATGTACGTTTTTC
Stk1T149ADir	CAAAAAGGTGACGATGCGATTTTTCTATCC
Stk1T149Rev	GCATCTTGCCAAGCCAATGTACGTTTTTC
GraRDir	GC*CATATG*ATGCAAATACTACTAGTAGAAGATGACAATAC
GraRRev	ACG*AAGCTT*TTATTATTCATGAGCCATATATCCTTTTCCTA
GraR^{C}Dir	GC*CATATG*GCTGAAGAAAAACGTACATTGACTTGG
GraR^{C}Rev	CG*AAGCTT*TTATTCATGAGCCATATATCCTTTTCC
vraFGDir	GC*GAATTC*CCCCATTACAAAATGAAATTGTTG
vraFGRev	GC*GGATCC*CACTCCTATAATTTATCTTAACTTC
Dir*P*graXSR	GCTAG*GATCCC*GCTAACATTGAAATGAAATTTTC
Rev*P*graXSR	GCTA*CATATG*CTAAAATACTCCTTTAAACTGTAAC
DirgraR-PMK4	GCTA*CATATG*CAAATACTACTAGTAGAAGATGAC
RevgraR-PMK4	GCTA*GTCGAC*TTATTCATGAGCCATATATCCTT

[a]The restriction sites are shown in italic.

Nano Liquid Chromatography (LC)−MS/MS of Phosphorylated GraR. The GraR samples were digested with trypsin (Promega, Madison, WI, USA) overnight at 37 °C in 100 mM ammonium bicarbonate and desalted using C18 ZipTips (Millipore, Billerica, MA, USA). The samples were then analyzed by nano LC−MS/MS on a NanoLC-Ultra 2D HPLC system coupled to an AB SCIEX 5600 hybrid quadrupole−quadrupole time-of-flight tandem mass spectrometer (Concord, Canada). The data acquisition cycle consisted of a single MS scan followed by MS/MS scans of the 10 most abundant ion peaks present in the MS spectrum.

Peptides and phosphopeptides were identified from the MS/MS data using the ProteinPilot 2.0.1 software package (AB SCIEX). The search conditions used for the sequence assignment were as follows: no cysteine modification, trypsin digestion, phosphorylation emphasis, biological modifications, amino acid substitutions, thorough identification, and a confidence threshold of 95% for protein detection. Spectra were searched against the entire UniProt protein sequence library (downloaded on September 8, 2012).[26]

Electrophoretic Mobility-Shift Assays (EMSA). The *vraRG* promoter region (P*vraFG*) spanning the region from −168 to +28 (relative to the transcription starting point) was amplified from the *S. aureus* Mu50 genome (Table 1) and used to assess the effect of GraR phosphorylation on the DNA-binding activity of GraR by EMSA. Target DNA was 5′-end labeled with γ-^{32}P-ATP using T4 kinase. Phosphorylation of GraR (25 μM) by Stk1 (5 μM) was performed in the presence of 500 μM ATP for 40 min at room temperature. As a control, GraR in the absence of Stk1 was incubated under the same conditions. Next, phosphorylated and unphosphorylated GraR were diluted at different concentrations using the EMSA buffer. The mixtures were supplemented with 4 ng of P*vraFG* and incubated for 30 min at room temperature. The reaction mixtures were resolved by 6% native PAGE. The gels were imaged using TYPHOON Trio^{+}. The phosphor images were analyzed using NIH ImageJ, version 1.45s.

Complementation of RN6390 Δ*graR* Strain with Wild-Type *graR* or Its Variants. To trans-complement the RN6390 Δ*graR* mutant strain[20] with *graR* or its variants, the following plasmids using the pMK4 shuttle vector (Bacillus Genetic Stock Center, Columbus, OH, USA) were constructed. The entire *graXRS* promoter region and *graR* were amplified from RN6390 (the primer sequences are provided in Table 1) and ligated together so that the promoter region is upstream of *graR*. This construct was cloned to the BamH1 and SalI restriction sites of the pMK4 vector. The pMK4::*graR* vector was amplified in *E. coli* NovaBlue; selection for the vector was carried out by growing *E. coli* in the presence of 100 μg/mL of ampicillin. The sequence of the construct was verified by DNA sequencing (The Center for Applied Genomics, Toronto, Canada). Before introducing pMK4::*graR* to the 6390 strain, the shuttle vector was introduced to *S. aureus* RN4220 using electroporation; chloramphenicol at 20 μg/mL was used to select for *S. aureus* harboring the pMK4::*graR* construct. The Δ*graR* strain complemented with wild-type graR was referred to as Δ*graR*^{comp}. Trans-complementation in the Δ*graR* mutant strain was confirmed by qRT-PCR, by monitoring the growth of complemented strain, and by determination of the minimum inhibitory concentration of vancomycin. The Δ*graR* mutant strain was also complemented with variants of *graR*, *graR*T130A/T149A and *graR*T128A/T130A/T149A. The respective strains were referred to as Δ*graR*^{comp}*graR*T130A/T149A (ΔgraR^{comp}*graR*-DM) and Δ*graR*^{comp}*graR*T128A/T130A/T149 (ΔgraR^{comp}*graR*-TM).

Nuclear Magnetic Resonance Spectroscopy (NMR) Experiments. To characterize the effect of mutations of Stk1 phosphorylation sites on WTA chemical composition, we isolated WTA from RN6390, Δ*graR*, Δ*graR*^{comp}, ΔgraR^{comp}*graR*-DM, ΔgraR^{comp}*graR*-TM, and Δ*vraG*[20] strains and studied their chemical composition by NMR. WTA was isolated from the above strains using trichloroacetic acid as described earlier.[27] To prevent the loss of plasmid during the growth of ΔgraR mutant and complemented ΔgraR mutants, chloramphenicol, at 10 μg/mL, was added into the Terrific Soy Broth media (TSB).

The isolated wall teichoic acid was lyophilized and resuspended in 100% D_2O to a 5−10 mg/mL concentration and placed in a 3 mm NMR tube. One-dimensional ^1H and 2D NMR spectra were collected at 25 °C on a Bruker AV III 700 MHz spectrometer (operating frequencies of 700.28 MHz for ^1H NMR and 176.096 MHz for ^{13}C NMR). The spectrometer was controlled with TOSPIN version 3.2 software and equipped with a 5 mm $^1H/^{13}C/^{15}N$ cryoprobe. For quantitative 1D ^1H spectra, 1D ^1H T_1 analysis was completed using the inversion recovery experiment.

RESULTS

Expression and Purification of the Target Protein. Stk1 is a membrane-bound protein with a single membrane-spanning domain. To aid purification, the protein was tagged with a six-histidine tag at its N-terminus, and 0.5% Triton X-100 was included in all of the buffers. The protein was purified to 90% homogeneity as assessed by Coomassie blue staining. The other proteins, Stp1, GraR, $GraR^N$, and Gra^C, are cytosolic and highly soluble. These proteins were purified to complete homogeneity and in high yields.

A triple-mutant GraR was constructed with residues Thr128, Thr130, and Thr149 in wild-type GraR replaced by alanine. The expressed protein formed insoluble species in the cells, indicating that mutation of these sites to alanine may affect the overall structure of GraR. We attempted to increase the solubility of the protein via several strategies, including lowering the temperature, altering isopropyl-β-D-thiogalactopyranoside (IPTG) concentration, or growing cells in the presence of D-sorbitol and β-alanine; however, we obtained unsuccessful results. We were, however, able to secure a small quantity of the soluble protein at 50% purity. A single point mutation at position 130 to alanine did not affect the expression or solubility of the protein.

GraR Undergoes Phosphorylation by Stk1. In our studies, *S. aureus* Stk1 underwent autophosphorylation with a rate constant of 0.10 ± 0.02 s^{-1} (Figures 2 and S1), and Stp1

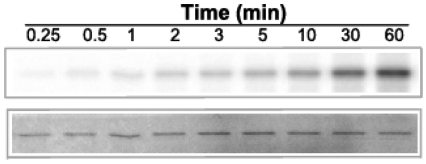

Figure 2. Autophosphorylation activity of Stk1. (A) Stk1 (2 μM) was incubated with [γ-^{32}P]-ATP (20 μM final concentration) at various time intervals in PB at room temperature. The samples were resolved by 12.5% SDS-PAGE. The top panel represents a phosphor-image of the gel, and the bottom panel shows Coomassie blue staining.

dephosphorylated Stk1 rapidly (Figure S2). Incubation of Stk1 with GraR resulted in phosphorylation of GraR (Figure 3A,B). These phosphorylated species were the target of Stp1 (Figure S2). To assess GraR phosphorylation by Stk1, we performed time-dependent phosphorylation. GraR underwent phosphorylation with a rate constant of 0.05 ± 0.01 s^{-1} (Figure 3C). To investigate whether the phosphorylation of GraR by Stk1 was specific, we thermo-denatured GraR and incubated it with Stk1 in the presence of ATP. The denatured GraR did not undergo phosphorylation by Stk1 (Figure 3D).

To assess further the specificity of the phosphotransfer between Stk1 and GraR, we investigated other response regulators, such as BceR and VraR. BceR is a homologue

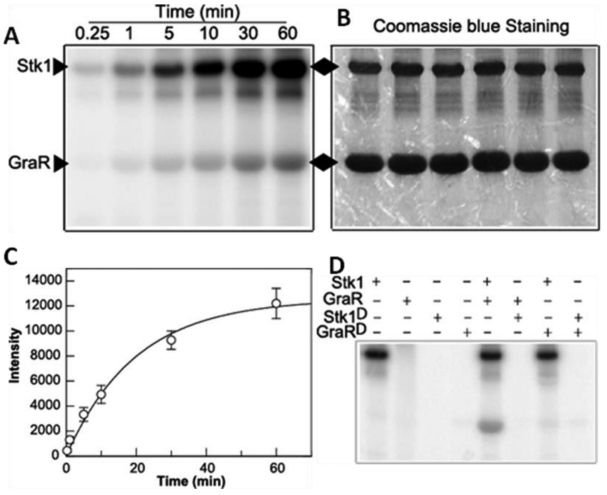

Figure 3. The kinase activity of Stk1. (A) GraR (25 μM) was incubated with Stk1 (10 μM) in the presence of 20 μM [γ-^{32}P]-ATP in PB at room temperature. Reactions were quenched with 5× SDS-PAGE sample buffer at various time intervals. This a phosphor scan of the gel. (B) Coomassie staining of the gel in panel A. (C) GraR time-dependent phosphorylation data from three independent experiments (errors for each data point represent the standard deviations). (D) Native (Stk1) or denatured ($Stk1^D$) kinase (5 μM) was incubated with 20 μM of the native (GraR) or denatured GraR ($GraR^D$) in the presence of 20 μM ATP for 30 min under the conditions described in panel A.

protein of GraR involved in bacitracin resistance in *B. subtilis*. Like GraSR, BceSR relies on an ABC transporter to confer resistance to bacitracin.[29] Sequence alignment of the two proteins indicates a 56% sequence identity (Figure 4). Despite this sequence identity, BceR did not undergo phosphorylation by Stk1 (Figure 5A). VraR is part of the *S. aureus* VraSR TCS involved in coordinating the cell-wall stimulon.[30] This protein belongs to the NarL class of response regulators.[31] Only the first 60 N-terminal amino acids of VraR share 22% similarity with the GraR primary sequence. Our experiments showed that VraR did not undergo phosphorylation by Stk1 (Figure 5A).

To determine the site of Stk1 phosphorylation on GraR, we cloned and purified the receiver and the effector domains of GraR as untagged proteins. $GraR^N$ spans residues 1−134, and $GraR^C$ spans residues 123−224. Only the C-terminal domain of GraR underwent phosphorylation by Stk1 (Figure 5B).

Tandem Mass Spectrometry (MS/MS) Determined the Putative Stk1 Phosphorylation Sites on GraR as Thr128, Thr130, and Thr 149. GraR alone, in the presence of ATP, or incubated with Stk1 in the presence of ATP was subjected to trypsin digestion. Tryptic peptides were investigated by MS/MS for phosphorylation. Coverage of the GraR sequence obtained by MS/MS exceeded 98% in all of the samples analyzed and in all cases. The observed precursor ion masses were within 0.05 Da of their calculated values, and the vast majority of product ion masses were within 0.003 Da of their calculated values. Phosphorylation was not evident in the samples containing only GraR or GraR with ATP (Figure 6A,C). Two singly phosphorylated tryptic peptides from GraR were identified with 99% confidence in the replicate preparations of GraR samples incubated with Stk1 in the presence of ATP. The sequences of the two phosphopeptides corresponded to amino acid residues 128−140 (TLTWQDAVVDLSK) and 146−154 (GDDTIFLSK) of the full-length protein (Figure 6B,D). Both peptides contain multiple potential sites (T and S) for

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                10        20        30        40        50        60
BceR  MFKLLLIEDDESLFHEIKDRLTGWSYDVYGIQDFSQVLQEFAAVNPDCVIIDVQLPKFDG-60
GraR  MQI-LLVEDDNTLFQELKKELEQWDFNVAGIEDFGKVMDTFESFNPEIVILDVQLPKYDG-59

BceR  FHWCRLIRSRSNVPILFLSSRDHPADMVMSMQLGADDFIQKPFHFDVLIAKIQAMFRRVH-120
GraR  FYWCRKMREVSNVPILFLSSRDNPMDQVMSMELGADDYMQKPFYTNVLIAKLQAIYRRVY-119

BceR  HYNTEPS-TIKTWCGAAVDAEQNLVSNDKGSVELTKNEMFILKQLIEQKNKIVSREELIR-179
GraR  EFTAEEKRTL-TWQDAVVDLSKDSIQKGDDTIFLSKTEMIILEILITKKNQIVSRDTIIT-178
                 *  *                    *

BceR  SLWNDERFVSDNTLTVNVNRLRKKLDALQLGAYIETKVGQGYIAKEEDKFYD-230
GraR  ALWDDEAFVSDNTLTVNVSRLRKKLSEISMDSAIETKVGKGYMAHE-224
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Figure 4. Sequence alignment of GraR and BceR. Highlighted in red and green are identical and similar residues, respectively. The Stk1 phosphorylation sites are indicated by asterisks.

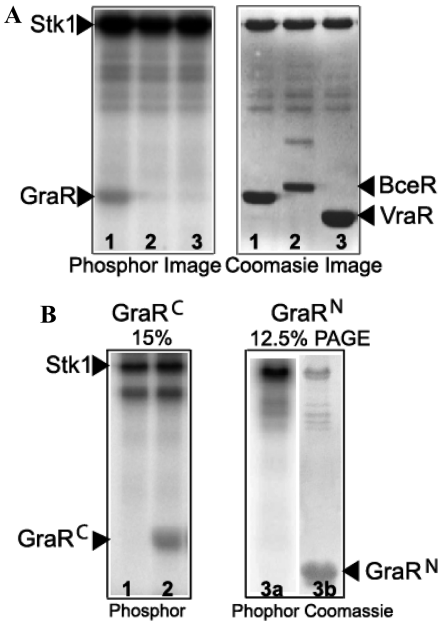

Figure 5. Specificity of GraR phosphorylation by Stk1. (A) Stk1 (5 μM) was incubated with GraR (10 μM; lane 1), BceR (10 μM; lane 2), or VraR (20 μM; lane 3) in the presence of 20 μM [γ-^{32}P]-ATP in PB for 30 min at room temperature. Samples were resolved by SDS-PAGE. (B) Stk1 (5 μM; lane 1) was incubated with GraR^{C} (20 μM; lane 2) or GraR^{N} (20 μM; lane 3a and 3b) under the same conditions as in panel A. Lanes 1−3a are the phosphor-image of the gel, and lane 3b is a Coomassie blue stain of lane 3a.

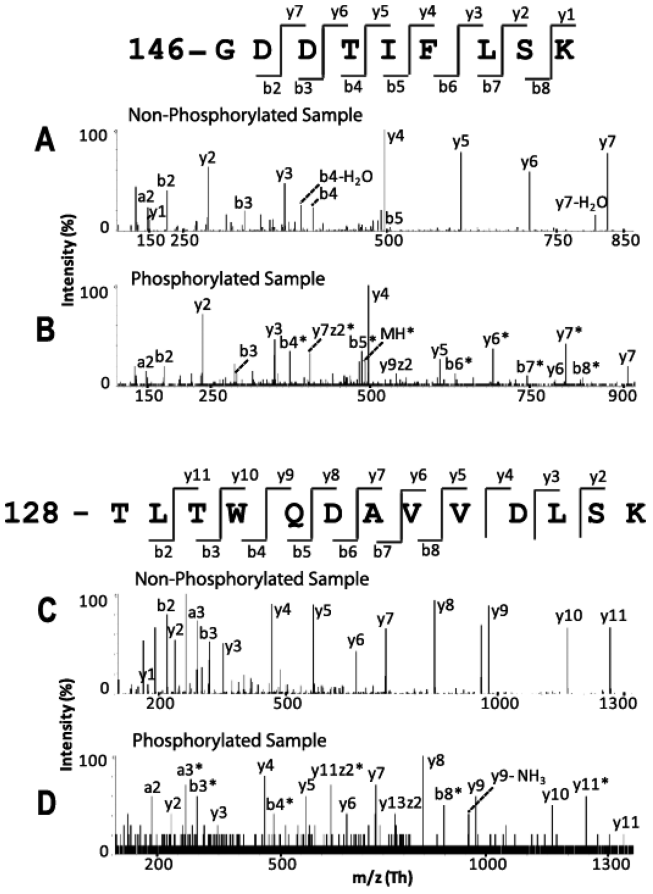

Figure 6. MS/MS spectra of tryptic GraR peptides containing the Stk1 phosphorylation sites. Sequence coverage is illustrated by the labeling of fragment ions of each peptide according to Roepstorff−Fohlmann−Biemann nomenclature. (A) MS/MS spectrum of the peptide 146-GDDTIFLSK-154 from untreated GraR. (B) MS/MS spectrum of the phosphopeptide 146-GDDtIFLSK-154 from GraR incubated with ATP in the presence of Stk1. (C) MS/MS spectrum of the peptide 128-TLTWQDAVVDLSK-140 from untreated GraR. (D) MS/MS spectrum of the phosphopeptide 128-TLtWQDAVVDLSK-140 from GraR incubated with ATP in the presence of Stk1 (lowercase letters indicate the phosphoamino acids).

phosphorylation. Definitive phosphorylation sites were unambiguously identified by MS/MS that produced characteristic product ion series to Thr128 and Thr130 in the peptide TLTWQDAVVDLSK and Thr149 in GDDTIFLSK (Table 2). MS/MS conclusively determined that Thr128 and Thr130 were simultaneously phosphorylated (Figure S3D and Table S1). The ions derived from the doubly phosphorylated peptide TLTWQDAVVDLSK suffered from low abundance. This was due to two mains factors. First, phosphorylation intrinsically decreases the efficiency with which a peptide ionizes in positive ion mode[32] and second, two further modifications were observed in this peptide that changed the mass of the sequence[33,34] and resulted in partitioning of this peptide among eight ion species, tryptophan oxidation and glutamine deamidation, which are two common, substoichiometric, and uncontrollable reactions in LC−MS experiments. To increase the sensitivity of the instrument for this species, we performed high-resolution multiple-reaction monitoring (MRMhr). This resulted in the increase of the signal-to-noise ratios between 1500 to 1 and 3745 to 1.

To confirm further that both threonines at positions 128 and 130 undergo phosphorylation, we constructed the GraR-Thr130Ala mutant. The MS/MS analysis indicated that Thr128 of GraR-Thr130Ala undergoes phosphorylation (Figure S3C and Table S1), confirming that both of these sites, in addition to Thr149, are targets for phosphorylation by Stk1. No phosphorylation was evident in the triple mutant treated with Stk1

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Table 2. MS/MS Analyses of the Target Peptides

ion	observed monoisotopic m/z (Th)	error (Th)	ion	observed monoisotopic m/z (Th)	error (Th)
GDDTIFLSK, 2+	Figure 6A		a3	228.1353	−0.0565
a2	145.0611	0.0003	b3	316.1869	0.0002
y1	147.1131	0.0003	y3	347.2306	0.0017
b2	173.0560	0.0003	y4	462.2572	0.0014
y2	234.1457	0.0009	b4-H_2O	484.2552	−0.0002
b3	288.0839	0.0013	y5	561.3251	0.0008
y3	347.2300	0.0012	$y10^{2+}$	580.8023	0.0014
b4-H_2O	371.1213	0.0016	b5	630.3246	<0.0001
b4	389.1316	0.0013	y6	660.3937	0.0010
M+2H-H_2O^{2+}	489.2527	0.0021	M+2H-H_2O^{2+}	729.3871	0.0017
y4	494.2972	−0.0001	y7	731.4308	0.0010
$M+2H^{2+}$	498.2563	0.0008	$M+2H^{2+}$	738.3908	0.0003
y5	607.3818	0.0004	b7	798.3788	0.0007
y6	708.4297	0.0006	y8	846.4583	0.0016
y7-H_2O	805.4465	0.0011	b8-H_2O	897.4438	−0.0027
y7	823.4566	0.0006	b8	915.4359	0.0212
GDDtIFLSK, 2+	Figure 6B		y9-NH_3	957.4907	0.0017
a2	145.0667	0.0059	y9	974.5158	0.0005
y1	147.1155	0.0027	y10	1160.5967	0.0021
b2	173.0548	−0.0009	y11	1261.6462	0.0039
y2	234.1452	0.0004	TLtWQDAVVDLSK, 2+	Figure 6D	
b3	288.0808	0.0018	a2	187.1497	0.0056
y3	347.2307	0.0018	y2	234.1386	−0.0062
b4-H_3PO_4	371.1197	<0.0001	a3-H_3PO_4	270.1226	−0.0586
y7-H_3PO_4 $^{2+}$	403.2272	0.0008	b3-H_3PO_4	298.1762	0.0001
b5-H_3PO_4	484.2046	0.0008	y3	347.2287	−0.0002
M+2H-$H_3PO_4^{2+}$	489.2523	0.0017	a4-H_3PO_4	456.2486	0.0119
y4	494.2996	0.0023	y4	462.2569	0.0011
$M+2H^{2+}$	538.2377	−0.0026	b4-H_3PO_4	484.2588	0.0034
y5	607.3747	0.0067	y5	561.3211	−0.0032
b6-H_3PO_4	631.2717	−0.0005	a5-H_3PO_4	584.2653	0.0538
y6-H_3PO_4	690.4173	−0.0012	$y11^{2+}$-H_3PO_4	622.3277	0.0082
b7-H_3PO_4	744.3632	0.0069	y6	660.3964	0.0037
y6	778.4078	0.0124	y7	731.4267	0.0031
y7-H_3PO_4	805.4371	−0.0083	$M+2H^{2+}$	778.3737	−0.0002
b8-H_3PO_4	831.4328	0.0445	y8	846.4567	<0.0001
y7	903.4244	0.0021	b8-H_3PO_4	897.4581	0.0116
TLTWQDAVVDLSK, 2+	Figure 6C		y9-NH_3	957.4901	−0.0013
y1	147.1137	0.0009	y9	974.5103	−0.0050
W	159.0920	0.0003	y10	1160.6106	0.0160
b2	215.1390	<0.0001	y11-H_3PO_4	1243.6186	0.0131
y2	234.1462	0.0014			

and ATP (Figure S3A,B), independently confirming that phosphorylation can only occur on the threonine residues at these sites and does not occur on either of the serine residues. The three threonine-to-alanine substitutions present in the GraR triple mutant were identified with 99% confidence or higher in multiple MS/MS spectra obtained from the mutant sample (Table S1).

Phosphorylation by Stk1 Increases the DNA-Binding Activity of GraR. To determine the amount of GraR phosphorylation in our experiments, we compared the radiolabeled band of GraR to that of Stk1 in the phosphotransfer experiments (Figure 1) using NIH ImageJ software and normalized against GraR concentration. At least four phosphorylation sites have been reported in STKs,[35] and three phosphorylation sites were observed in GraR. By normalizing for the proteins concentrations in our assay (Figure 5), we determined that about 9% of GraR undergoes phosphorylation (corroborated by SDS-phostag-PAGE,[36] data not shown). The low level of phosphorylation of GraR by Stk1 observed in our experiments could be due to our experimental settings or could be an indication that quantitative phosphorylation of GraR by Stk1 may depend on the activation of GraR by GraS, a phosphorylation process that results in phosphorylation of the conserved Asp-51 in the N-terminus of GraR. It is possible that phosphorylation by GraS may expose further the Stk1-phosphorylation sites on GraR. Our efforts to phosphorylate GraR by small-molecule donors such as acetyl phosphate were not successful.

The EMSA experiments indicated an increase by 1.5-fold in the binding affinity of phosphorylated GraR to the *vraFG* promoter region (Figure 7). Taking into account that in the EMSA experiments ∼9% of GraR is phosphorylated, the effect of Stk1-phosphorylation on the DNA-binding activity of GraR is significant.

Role of Stk1 Phosphorylation of GraR Functions in *S. aureus*. The Δ*graR* mutant exhibited a regular growth in

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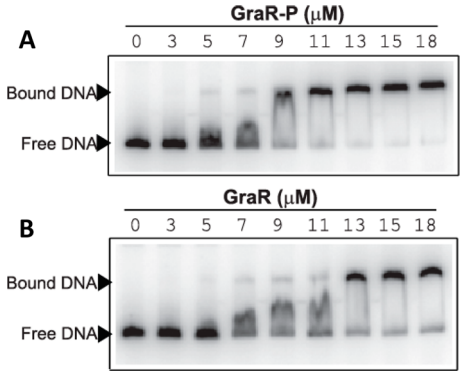

Figure 7. Effect of phosphorylation on GraR binding to P*vraFG*. (A) GraR (25 μM) was incubated with 5 μM Stk1 in the presence of 500 μM ATP for 30 min, and the mixture then was desalted. GraR at different concentrations was incubated with ^{32}P-5′-end labeled P*vraFG* (4 ng) for 30 min. Samples were resolved by 6% native PAGE. (B) GraR (25 μM) alone was incubated with 500 μM ATP and desalted. Different concentrations of GraR were incubated with ^{32}P-5′-end labeled P*vraFG* (4 ng). Samples were resolved by 6% native PAGE.

TSB for up to 4 h followed by stagnated growth as assessed by measurement of the OD at 600 nm and by viability test on TSB-agar plates (data not shown). Deletion of *graR* decreased the MIC of vancomycin 2-fold (MIC$^{\Delta graR}$ = 0.5 μg/mL and MIC^{RN6390} = 1 μg/mL). Complementation of Δ*graR* with wild-type *graR*, *graR*DM, and *graR*TM restored the normal growth of the bacteria and the MIC of vancomycin. The qRT-PCR studies showed that the pMK4::*graR* vector complemented the *graR* expression when compared to the wild-type strain RN6390.

To investigate the biological role of Stk1 phosphorylation of GraR in *S. aureus*, we analyzed WTA isolated from RN6390 by NMR spectroscopy and compared it with WTA isolated from Δ*graR*, Δ*graR*^{comp}, Δ*graR*^{comp}*graR*DM, Δ*graR*^{comp}*graR*TM, and Δ*vraG*. The following experiments were acquired for each sample. Quantitative 1D proton, 2D ^{1}H−^{13}C HSQC, and 2D ^{1}H−^{13}C HSQC decoupled. In addition, 2D ^{1}H−^{13}C HSQC-DEPT, 2D ^{1}H−^{13}HSQC-TOCSY, 2D ^{1}H−^{13}C HMBC, 2D ^{1}H−^{1}H TOCSY, and 2D ^{1}H−^{1}H COSY spectra were acquired for sample T128/7149 Δ GraR. ^{1}H NMR of WTA isolated from RN6390 had all of the resonances previously reported for WTA of *S. aureus* RN4220 as described by Xia et al. and Bernal et al. (Figure 8),[27,28] including the resonances consistent with both configurations of anomeric protons Hα and Hβ at δ 4.95 and 4.65 ppm, respectively. The 2D ^{1}H−^{13}C HSQC-DEPT spectrum enabled differentiation between methylene and methine resonances from the ribitol and GlcNAc moieties.

The ^{1}H NMR signals at δ 2.10 and 1.63 ppm (Figure 8) correspond to the protons in the N-acetyl (NAc) group of the N-acetylglucosoamine (GlcNAc) and D-Ala groups, which modify the C4-OH and C2-OH of ribitol, respectively. The ^{1}H NMR signals for ribitol and GlcNAc were clustered in the region 4.2−3.85 ppm as reported earlier.[27,28] Comparison of the ^{1}H NMR spectra obtained from the various samples of WTA indicated that the relative intensity of the NAc and D-Ala proton resonances varied significantly. By using the area under the resonance range of ribitol and GlcNAc protons (4.20−3.85) as a reference integral, the relative amounts of NAc and D-Ala were calculated as shown in Figure 8.

Deletion of *graR* or *vraG* had a major effect on the D-Ala content of WTA, with Δ*vraG* having the largest effect (Figure 8).

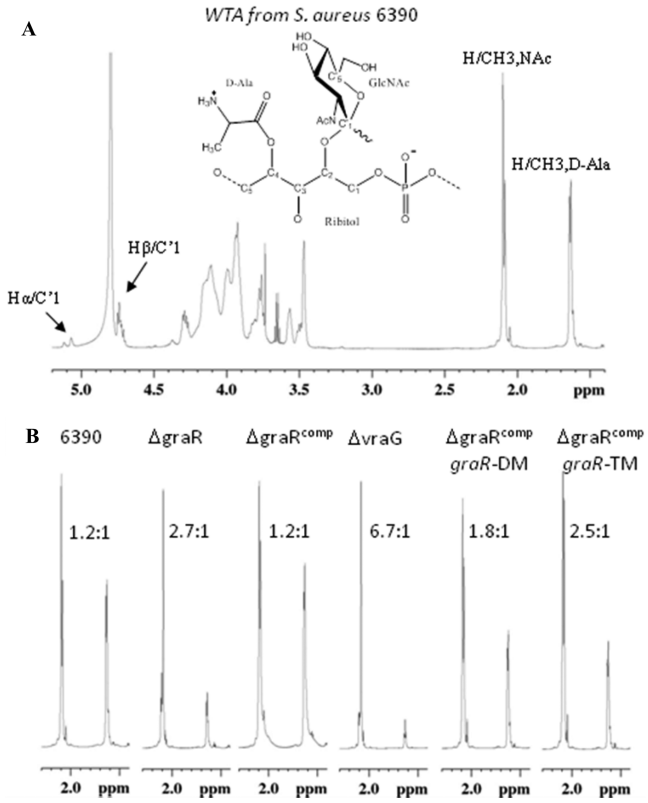

Figure 8. (A) One-dimensional ^{1}H NMR spectrum of WTA isolated from RN6390. The inset is the structure of the WTA monomer, ribitol-5-phosphate modified by D-Ala at C2, and N-acetyl glucosoamine (GlcNAc) at C4. (B) Snap-shots of the 1D proton NMR spectra of WTA isolated from Δ*vraG*, Δ*graR*, Δ*graR*^{comp}, Δ*graR*^{comp}*graR*-DM, and Δ*graR*^{comp}*graR*-TM highlighting the region of H/CH3 from the NAc group of GlcNAc and D-Ala.

These observations are in agreement with the observations reported by Meehl et al., whereby deletion of *graRS* or *vraFG* resulted in an increase in the negative net surface of the respective mutant strains.[20] The similarity in behavior between the *graR* and *vraG* mutants is also in agreement with the report by Yang et al., which demonstrated that deletions of *vraG* and *graR* result in a reduction of the expression levels of *dltA*.[37]

The reduction in D-Ala modification of WTA was reversed in the wild-type *graR*-complemented Δ*graR* strain. However, mutations of the Stk1 phosphorylation sites in GraR did not restore this phenotype completely, with the triple mutant exhibiting similar levels of reduction in D-Ala content as the Δ*graR* mutant. This suggests that phosphorylation of GraR by Stk1 may modulate the transcriptional regulatory activity of GraR to *dltA*.

DISCUSSION

Stk1 is implicated in cell-wall metabolism, virulence, and resistance to cell-wall inhibitors.[14,18] However, substrates of Stk1 remain elusive in *S. aureus*. STKs and STPs from other organisms have been involved in the regulation of gene expression through reversible phosphorylation of response regulator proteins and components of the transcription and translation machinery as well as in alteration of metabolism through post-translational modification of enzymes.[38] In particular, in *S. aureus*, Stk1 has been shown to phosphorylate the global transcription factors SarA[39,40] and MrgA[41−43] as well as glycolytic enzymes.[44]

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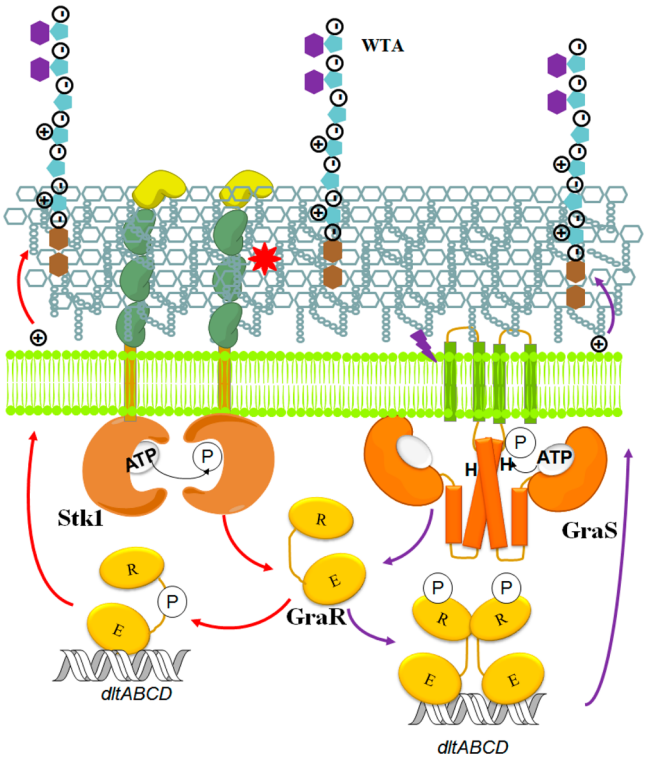

Figure 9. Illustration of the proposed crosstalk between Stk1/Stp1 and GraSR. Each signaling pathway may alter the cell-wall charge in response to different environmental cues. The negatively charged phosphate groups in WTA are represented by circles with the "−" symbol in them, ribitols are represented by cyan pentagons, D-Ala groups in WTA are represented by circles with the "+" symbol in them, and GlcNAc groups are represented by purple hexagons. The expression of the *dltABCD* operon is modulated by two independent phosphorylation processes mediated by Stk1 or GraS depending on the environmental cues.

Our study shows that GraR undergoes specific phosphorylation at the DNA-binding domain by Stk1 (Figure 9). Phosphorylation is dependent on the intact tertiary structure of GraR, as denatured GraR does not undergo phosphorylation. Analysis of the GraR tryptic peptides by MS/MS identified unambiguously three potential phosphorylation sites, Thr128, Thr130, and Thr149. Absence of phosphorylation elsewhere in the GraR triple mutant suggests that these are the only sites phosphorylated by Stk1 in GraR.

The structural studies on OmpR-like RRs show that members share similar structural features.[45−47] Guided by the sequence similarities between GraR and OmpR and the 3D structure of OmpR,[45] we determined that the phosphorylation sites in GraR, Thr128, Thr130, and Thr149, reside in a putative four-stranded antiparallel β-sheet region of the GraR DNA-binding domain (Figure S4), a feature shared by all of the OmpR-like RRs.[45−47] $GraR^N$ (1−134) and $GraR^C$ (123−224) share the region from 123 to 134, and yet only $GraR^C$ underwent phosphorylation by Stk1, possibly because this region folds properly only in the context of the effector domain because it carries the conserved hydrophobic core residues of this domain.[45] This observation provides evidence for the specific recognition of GraR structural features by Stk1.

The specificity of GraR phosphorylation was further corroborated by the lack of phosphorylation of BceR and VraR, which belong to the OmpR and NarL families of response regulators, respectively. BceR is part of the BceR TCS in *B. subtilis*. This system mediates bacitracin resistance.[29,48] The primary sequence alignment (Figure 4) shows that two of the putative phosphorylation sites in GraR are present in BceR, namely, Thr128 and Thr130; however, Thr149 in GraR corresponds to a serine residue in BceR. Notably, the peptide sequence in which Thr128 and Thr130 reside in GraR differs in BceR; moreover, two positively charged amino acids that proceed Thr128 (Lys125-Arg126) are replaced by serine in BceR (Figure 4). These differences are likely key contributors to the absence of phosphorylation in BceR by Stk1. VraR is part of the TCS VraSR that mediates *S. aureus* response to cell-wall antibiotics. There is no sequence similarity between the effector domains of VraR and GraR, which may explain the lack of phosphorylation by Stk1.

A recent study by Sun et al. showed that SarA and MgrA undergo phosphorylation by Stk1 on a cysteine residue.[40] Notably, GraR lacks a cysteine residue in the effector domain (Figure 4) but has a cysteine residue in the receiver domain. However, only the effector domain underwent phosphorylation in our study. Furthermore, BceR has a cysteine residue in the effector domain in addition to in the receiver domain; however, the protein did not undergo phosphorylation. These observations provide strong evidence that the cysteine residue is not a phosphorylation site in GraR, and they indicate the diversity of the amino acids phosphorylated by Stk1.

NMR analysis of WTA isolated from the RN6390, Δ*vraG*, Δ*graR*, Δ$graR^{comp}$, Δ$graR^{comp}$*graR*-DM, and Δ$graR^{comp}$*graR*-TM strains revealed that Stk1 phosphorylation of GraR modulates the extent of the modification of WTA by D-Ala. The effect of mutations of the Stk1-phosphorylation sites of GraR on WTA was similar to that of deletion of *graR*. These observations suggest that modification of WTA by D-Ala may be modulated through the phosphorylation of GraR by Stk1.

The EMSA studies showed Stk1 phosphorylation of GraR enhanced the DNA-binding affinity of GraR to the *vraFG* promoter region by 1.5-fold, which agrees with a reported a 1.7- to 1.8-fold reduction in the *vraFG* expression for the *S. aureus* Δ*stk1* mutant.[11] The positive effect that Stk1 phosphorylation has on the GraR DNA-binding activity suggests that phosphorylation of GraR by Stk1 may also have a positive effect on regulation of the *dltABCD* operon. This hypothesis is supported well by our findings that mutations of the Stk1 phosphorylation sites on GraR result in the decrease of D-Ala modification of WTA. Modification of the DNA-binding activity of RRs by STKs has been reported for a few TCSs; among them, CovRS of group B streptococci has been studied extensively.[49,50] CovSR is involved in regulation of the virulence factors.[51] In this case, phosphorylation by Stk1 takes place in the receiver domain of CovR and represses the DNA-binding activity of CovR.[52]

The potential role of GraR phosphorylation by Stk1 could be to modify the charge of WTA in response to signals other than those sensed by the sensor GraS or to modulate signals that come through GraS. Notably, GraR does not regulate its own expression, unlike many other response regulators,[30,53] and its expression is not induced by the presence of CAMPs.[19] This is an indication that the function of GraR could be modulated through post-translational modification, such as phosphorylation by Stk1, independently of GraS.

WTA is involved in many processes such as biofilm formation,[54] cell growth and division,[55] infection and attachment of *S. aureus* to artificial surfaces and host tissues,[56−58] and

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antibiotic susceptibility.[54−56,59] These processes have been shown to depend on the WTA charge, which is modulated through the attachment of D-alanyl esters to the hydroxyls groups on WTA ribitols;[54,59] D-Ala adds a positively charged amine on WTA. The charge of WTA has also been implicated in spatial and temporal regulation of cell-wall modeling proteins such as penicillin binding protein 4 (PBP4),[60] the major autolysin in *S. aureus*, AtlA,[61] and the PBP-like protein FmtA.[62] Our findings that Stk1, a kinase that is involved in cell-wall metabolism, phosphorylates GraR, a transcription factor directly involved in regulation of D-Ala modification of WTA, suggest that processes linked to cell-wall remodeling may require modulation of the cell-wall charge. Post-translational regulation of GraR DNA-binding activity by Stk1 constitutes an economical regulatory mechanism because it links the cell-wall sensory system to the regulatory system of cell-wall charge.

Considering the role that eukaryote-like Ser/Thr kinases and cognate phosphatases play in virulence and antibiotic resistance in *S. aureus*, they are suitable targets for drug development.[18] Numerous STK inhibitors have been approved by the United States Food and Drug Administration for use in humans.[63] Understanding the functions of these proteins and their network is paramount to understanding the *S. aureus* stress response and examining signaling pathways as drug targets.

■ ASSOCIATED CONTENT

Supporting Information

Quantification of Stk1 autophosphokinase activity; phosphatase activity of Stp1; MS/MS spectra of tryptic GraR peptides obtained from Stk1-treated GraR mutant samples; X-ray crystal structure of *E. coli* OmpR; and MS/MS analyses of the target peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

TCS, two-component system; RR, response regulator; HK, histidine kinase; CAMP, cationinc antimicrobial peptide; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; ATP, adenine triphosphate; DTT, dithiothretiol; LC, liquid chromatography; MS, mass spectrometry; EMSA, electrophoretic mobility shift assay

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