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RESEARCH ARTICLE

Phosphorylation at the D53 but Not the T65 Residue of CovR Determines the Repression of *rgg* and *speB* Transcription in *emm*1- and *emm*49-Type Group A Streptococci

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ABSTRACT CovR/CovS is a two-component regulatory system in group A *Streptococcus* and primarily acts as a transcriptional repressor. The D53 residue of CovR ($CovR_{D53}$) is phosphorylated by the sensor kinase CovS, and the phosphorylated $CovR_{D53}$ protein binds to the intergenic region of *rgg-speB* to inhibit *speB* transcription. Nonetheless, the transcription of *rgg* and *speB* is suppressed in *covS* mutants. The T65 residue of CovR is phosphorylated in a CovS-independent manner, and phosphorylation at the D53 and T65 residues of CovR is mutually exclusive. Therefore, how phosphorylation at the D53 and T65 residues of CovR contributes to the regulation of *rgg* and *speB* expression was elucidated. The transcription of *rgg* and *speB* was suppressed in the strain that cannot phosphorylate the D53 residue of CovR ($CovR_{D53A}$ mutant) but restored to levels similar to those of the wild-type strain in the $CovR_{T65A}$ mutant. Nonetheless, inactivation of the T65 residue phosphorylation in the $CovR_{D53A}$ mutant cannot derepress the *rgg* and *speB* transcription, indicating that phosphorylation at the T65 residue of CovR is not required for repressing *rgg* and *speB* transcription. Furthermore, *trans* complementation of the $CovR_{D53A}$ protein in the strain that expresses the phosphorylated $CovR_{D53}$ resulted in the repression of *rgg* and *speB* transcription. Unlike the direct binding of the phosphorylated $CovR_{D53}$ protein and its inhibition of *speB* transcription demonstrated previously, the present study showed that inactivation of phosphorylation at the D53 residue of CovR contributes dominantly in suppressing *rgg* and *speB* transcription.

IMPORTANCE CovR/CovS is a two-component regulatory system in group A *Streptococcus* (GAS). The D53 residue of CovR is phosphorylated by CovS, and the phosphorylated $CovR_{D53}$ binds to the *rgg-speB* intergenic region and acts as the transcriptional repressor. Nonetheless, the transcription of *rgg* and Rgg-controlled *speB* is upregulated in the *covR* mutant but inhibited in the *covS* mutant. The present study showed that nonphosphorylated $CovR_{D53}$ protein inhibits *rgg* and *speB* transcription in the presence of the phosphorylated $CovR_{D53}$ *in vivo*, indicating that nonphosphorylated $CovR_{D53}$ has a dominant role in suppressing *rgg* transcription. These results reveal the roles of nonphosphorylated $CovR_{D53}$ in regulating *rgg* transcription, which could contribute significantly to invasive phenotypes of *covS* mutants.

KEYWORDS CovR/CovS, Rgg, SpeB, group A *Streptococcus*

Streptococcus pyogenes (group A *Streptococcus* [GAS]) is a Gram-positive human pathogen causing diseases from mild pharyngitis and tonsillitis to severe cellulitis, necrotizing fasciitis, and toxic shock syndrome. An increase in invasive GAS infections

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has been reported globally (1–6) and is associated with high mortality and economic burden (7, 8). Therefore, there is an urgent need to understand the pathogenesis of invasive GAS infections.

Mutations in *covS* and *covR* genes are detected more frequently in strains isolated from patients with severe manifestations than from patients with mild symptoms (9–12). CovR/CovS is a two-component regulatory system of GAS (13). CovS acts as a membrane-associated sensor kinase/phosphatase to modulate the phosphorylation levels of the intracellular response regulator CovR (14, 15). There are two residues of CovR that can be phosphorylated. The phosphorylation level of the D53 residue of CovR ($CovR_{D53}$) is modulated by CovS; however, the T65 residue of CovR ($CovR_{T65}$) is phosphorylated by a CovS-independent mechanism (16). In addition, *in vitro* analysis showed that phosphorylation of these two residues in the recombinant CovR protein is mutually exclusive (16). Inactivation of CovS results in decreased levels of phosphorylated $CovR_{D53}$ and the derepression of CovR-regulated virulence factors, including the hyaluronic acid capsule synthesis (*has* operon), streptolysin S (*sls*), streptolysin O (*slo*), NADase (*nga*), and DNase (*sda1*) (14, 17–19). In addition, although CovS is considered to regulate target genes solely through CovR (20), a strain that expresses nonphosphorylated $CovR_{D53}$ is more virulent than the *covR* deletion strain in a mouse infection model (16), suggesting that nonphosphorylated $CovR_{D53}$ in the *covS* mutant still has important roles in GAS pathogenesis. Nonetheless, how CovR contributes to GAS pathogenesis in the absence of CovS is still not clear.

Rgg is a positive regulator of the cysteine protease SpeB (21, 22). The *emm*3-type isolates with mutations in *rgg* express a higher level of virulence factors, such as *slo*, *nga*, and *ska,* than do isolates with intact *rgg* (11). In the *emm*49-type NZ131 strain, Rgg acts as a transcriptional repressor of genes that are involved in virulence, metabolism, and oxidative stress responses (23–25). Both *rgg* mutants of *emm*3- and *emm*49-type strains showed higher lethality in the mouse infection models than did wild-type strains (11, 23), indicating that inactivation of Rgg contributes to the increase of bacterial virulence.

The phosphorylated $CovR_{D53}$ protein has better DNA-binding activity than the nonphosphorylated $CovR_{D53}$ protein (26–28). The phosphorylated $CovR_{D53}$ protein binds to the *rgg-speB* intergenic region to inhibit *speB* transcription (28–30). Nonetheless, the transcription of *rgg* is repressed in the *covS* mutant and therefore resulted in the inactivation of *speB* transcription (19, 30). These results indicate that the *covS* mutant and *covR* mutant are not identical. In addition, unlike the direct binding of the phosphorylated $CovR_{D53}$ protein and its inhibition of *speB* transcription, which have been extensively demonstrated previously, whether nonphosphorylated $CovR_{D53}$ is capable of repressing *rgg* transcription and subsequently preventing SpeB production has not been verified.

Phosphorylation of the CovR D53 and T65 residues is mutually exclusive (16). Inactivation of the CovR protein phosphorylation by a D53A or T65A amino acid substitution does not result in aberrantly folded protein *in vitro* (16). Nonetheless, the $CovR_{T65A}$ mutant was hypervirulent compared to the wild-type strain (16), suggesting that the phosphorylated $CovR_{T65}$ protein contributes significantly to the regulatory networks of GAS. The present study aims to analyze how phosphorylated $CovR_{D53}$ and $CovR_{T65}$ proteins mediate the repression of *rgg* and *speB* transcription. The results showed that phosphorylation at the T65 residue of CovR was not required for suppressing *rgg* and *speB* transcription. In addition, the nonphosphorylated $CovR_{D53}$ had the more dominant roles in inhibiting *rgg* and *speB* transcription than did the phosphorylated $CovR_{D53}$ protein.

RESULTS

Transcription of *rgg* was inhibited in the $CovR_{D53A}$ mutant but derepressed in the $CovR_{T65A}$ mutant. Phosphorylation of $CovR_{D53}$ promotes its dimerization and increases its activity to bind to the *rgg-speB* intergenic region (28). CovR acts as a transcriptional repressor of *rgg*; however, the deletion of *covS* and inactivation of the

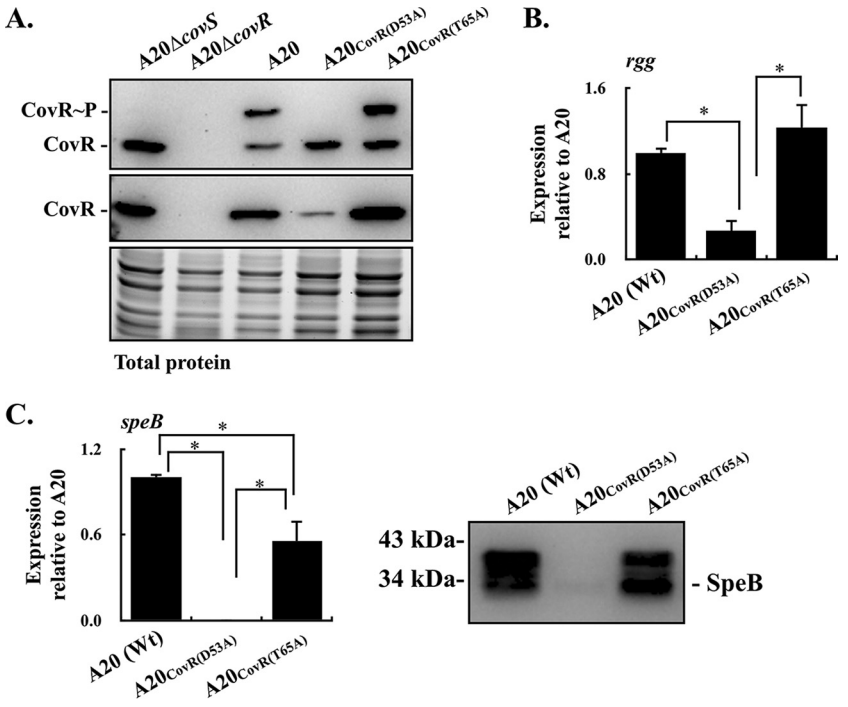

FIG 1 Phosphorylation levels of CovR and the expression of CovR, *rgg,* and *speB* in the wild-type A20 strain (*emm*1 type) and its $CovR_{D53A}$ and $CovR_{T65A}$ mutants. Bacterial strains were cultured to the exponential (for detecting CovR) and stationary (for detecting *rgg* and *speB*) phases of growth. RNAs, culture supernatants, and total proteins were collected for quantitative real-time PCR (RT-qPCR), Western blotting, and Phos-tag Western blot analyses. (A) Levels of phosphorylation and expression of CovR protein in A20, $CovR_{D53A}$ mutant, and $CovR_{T65A}$ mutant. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. Total protein serves as the loading control. (B) Transcription of *rgg* in A20, $CovR_{D53A}$ mutant, and $CovR_{T65A}$ mutant. (C) The transcription of *speB* and secretion of SpeB protein in A20, $CovR_{D53A}$ mutant, and $CovR_{T65A}$ mutant. Biological replicate experiments were performed using three independent preparations. The expression of *rgg* and *speB* was normalized to that of *gyrA*. Wt, wild-type strain; *, $P < 0.05$.

D53 residue phosphorylation of CovR result in the repression of *rgg* transcription (19, 30), suggesting that the repression is unrelated to the phosphorylation at D53. In addition to the D53 residue, the T65 residue of CovR is phosphorylated by a CovS-independent mechanism (16). Horstmann et al. (16) showed that phosphorylation at the D53 and T65 residues of CovR is mutually exclusive; therefore, the phosphorylated $CovR_{T65}$ could mediate the repression of *rgg* in the *covS* mutant. In order to elucidate whether the phosphorylated $CovR_{T65}$ is involved in the repression of *rgg* transcription, the isoallelic GAS strains that were unable to be phosphorylated at D53 [$A20_{CovR(D53A)}$ mutant] and T65 [$A20_{CovR(T65A)}$ mutant] were constructed, and the levels of CovR protein expression and phosphorylation in these mutants were analyzed by Western blotting and Phos-tag Western blotting, respectively. The results showed that the expression of CovR protein in the $CovR_{D53A}$ mutant was decreased compared to those in the wild-type A20 strain and $CovR_{T65A}$ mutant (Fig. 1A). The phosphorylated $CovR_{D53}$ protein was detected in the wild-type A20 strain and its $CovR_{T65A}$ mutant (Fig. 1A). Nonetheless, the phosphorylated $CovR_{T65}$ protein cannot be observed in the *covS* and $CovR_{D53A}$ mutants (Fig. 1A), suggesting that low levels of phosphorylated $CovR_{T65}$ were presented in those mutants or that the phosphorylated $CovR_{T65}$ cannot be detected by Phos-tag Western blotting effectively. Next, the transcription levels of *rgg* in the wild-type A20 strain, $A20_{CovR(D53A)}$ mutant, and $A20_{CovR(T65A)}$ mutant were analyzed. Compared to the wild-type A20 strain, the transcription of *rgg* was inhibited in the $A20_{CovR(D53A)}$ mutant (Fig. 1B). Nonetheless, the transcription of *rgg* in the $A20_{CovR(T65A)}$ mutant was restored to a level similar to that in the wild-type A20 strain (Fig. 1B).

Rgg is the positive regulator of streptococcal pyrogenic exotoxin B (SpeB) (21). Therefore, whether the expression of SpeB was derepressed in the $A20_{CovR(T65A)}$ mutant

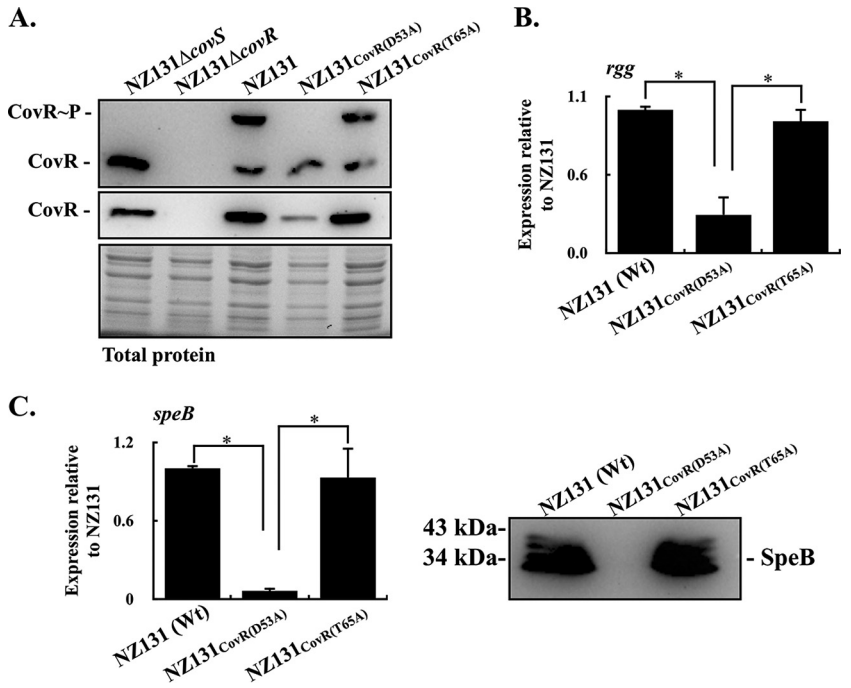

FIG 2 Phosphorylation levels of CovR and the expression of CovR, *rgg,* and *speB* in the wild-type NZ131 strain (*emm*49 type) and its $CovR_{D53A}$ and $CovR_{T65A}$ mutants. Bacterial strains were cultured to the exponential (for detecting CovR) and stationary (for detecting *rgg* and *speB*) phases of growth. RNAs, culture supernatants, and total proteins were collected for RT-qPCR, Western blotting, and Phos-tag Western blot analyses. (A) Levels of phosphorylation and expression of CovR protein in NZ131, $CovR_{D53A}$ mutant, and $CovR_{T65A}$ mutant. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. Total protein serves as the loading control. (B) Transcription of *rgg* in NZ131, $CovR_{D53A}$ mutant, and $CovR_{T65A}$ mutant. (C) Transcription of *speB* and secretion of SpeB protein in NZ131, $CovR_{D53A}$ mutant, and $CovR_{T65A}$ mutant. Biological replicate experiments were performed using three independent preparations. The expression of *rgg* and *speB* was normalized to that of *gyrA*. Wt, wild-type strain; *, $P < 0.05$.

was further analyzed with quantitative PCR (qPCR) and Western blotting. The results showed that the transcription of *speB* was partially restored in the $A20_{CovR(T65A)}$ mutant compared to the $A20_{CovR(D53A)}$ mutant (Fig. 1C). Furthermore, both the zymogen (42 kDa) and mature (28 kDa) forms of SpeB proteins were detected in the supernatants from the wild-type A20 strain and $A20_{CovR(T65A)}$ mutant (Fig. 1C). These results support the idea that the nonphosphorylated $CovR_{D53}$ acts as the repressor for *rgg* transcription; in addition, the phosphorylated $CovR_{T65}$ may also participate in the regulation of *rgg* transcription.

Inactivation of $CovR_{D53}$ phosphorylation in the *emm*49-type NZ131 strain inhibits *rgg* and *rgg*-controlled *speB* transcription. The results shown in Fig. 1 indicate that the transcription of *speB* was inhibited in the $A20_{CovR(D53A)}$ mutant but derepressed in the $A20_{CovR(T65A)}$ mutant. Nonetheless, in the *emm*3-type strain, the transcription of *speB* was derepressed both in $CovR_{D53A}$ and $CovR_{T65A}$ mutants (16). The *emm*3-type strain is rarely found in Taiwan. Therefore, to elucidate how $CovR_{D53A}$ and $CovR_{T65A}$ mutations influence the transcription of *rgg* and *speB*, the $CovR_{D53A}$ and $CovR_{T65A}$ mutants of another reference strain, NZ131 (*emm*49 type), were constructed, and the transcription of *rgg* and *speB* was analyzed. The levels of expression and phosphorylation of CovR proteins in NZ131 and its *covR* mutants were similar to those observed in A20 and its *covR* mutants (Fig. 2A). Compared to the wild-type NZ131 strain, the transcription of *rgg* and *speB* was repressed in the $NZ131_{CovR(D53A)}$ mutant but derepressed in the $NZ131_{CovR(T65A)}$ mutant (Fig. 2B and C). These results further support the idea that the nonphosphorylated $CovR_{D53}$ or phosphorylated $CovR_{T65}$ could mediate the repression of *rgg* and *speB* transcription.

Inactivation of $CovR_{T65}$ phosphorylation in the $CovR_{D53A}$ mutant cannot derepress *rgg* and *speB* transcription. In order to further verify whether phosphorylation

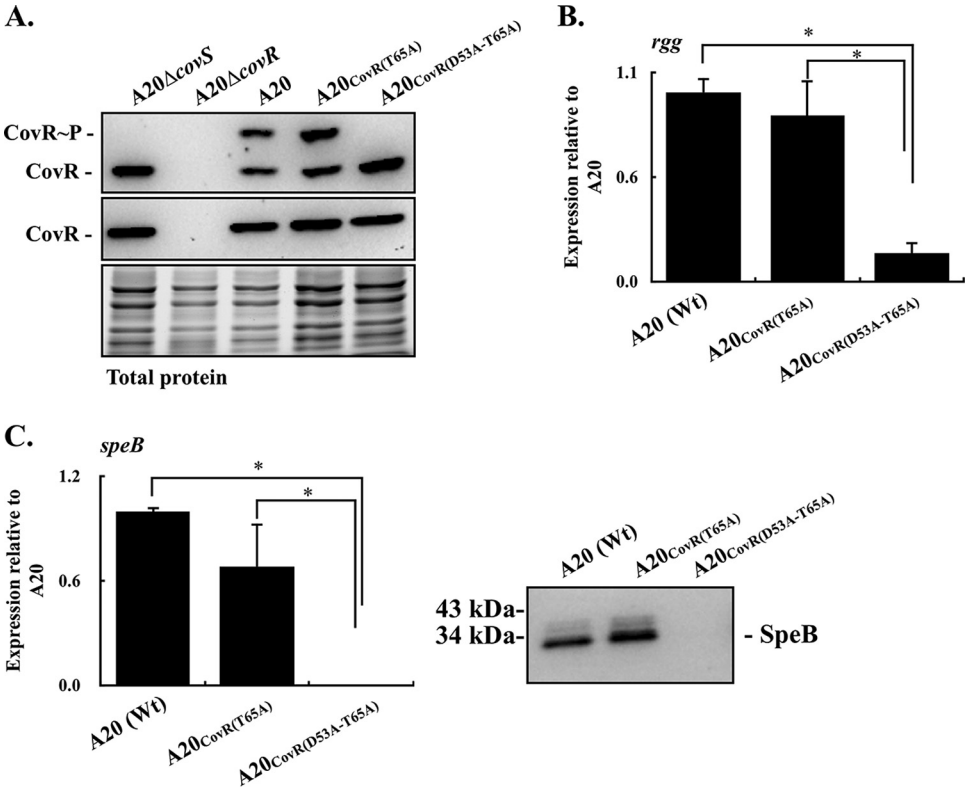

FIG 3 Phosphorylation levels of CovR and the expression of CovR, *rgg,* and *speB* in *emm*1-type A20 and its $CovR_{T65A}$ and $CovR_{D53A-T65A}$ mutants. Bacterial strains were cultured to the exponential (for detecting CovR) and stationary (for detecting *rgg* and *speB*) phases of growth. RNAs, culture supernatants, and total proteins were collected for RT-qPCR, Western blotting, and Phos-tag Western blot analyses. (A) Levels of phosphorylation and expression of CovR protein in A20, $CovR_{D53A}$ mutant, and $CovR_{D53A-T65A}$ mutant. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. Total protein serves as the loading control. (B) Transcription of *rgg* in A20, $CovR_{T65A}$ mutant, and $CovR_{D53A-T65A}$ mutant. (C) Transcription of *speB* and secretion of SpeB protein in A20, $CovR_{T65A}$ mutant, and $CovR_{D53A-T65A}$ mutant. Biological replicate experiments were performed using three independent preparations. The expression of *rgg* and *speB* was normalized to that of *gyrA*. Wt, wild-type strain; *, $P < 0.05$.

at the T65 residue of CovR is required for suppressing *rgg* and *speB* transcription, the T65A amino acid substitution of CovR in the $A20_{CovR(D53A)}$ mutant was constructed [$A20_{CovR(D53A-T65A)}$ mutant]. As expected, results from the Phos-tag Western blot analysis showed that the phosphorylated CovR proteins cannot be detected in the $A20_{CovR(D53A-T65A)}$ mutant (Fig. 3A). In addition, different from the A20 and its $CovR_{D53A}$ mutant, the expression level of CovR in $CovR_{T65A}$ and $CovR_{D53A-T65A}$ mutants was similar (Fig. 1A and 3A). Although the phosphorylated $CovR_{T65}$ protein in the $CovR_{D53A}$ mutant cannot be detected by Phos-tag Western blotting, these results support the idea that the phosphorylated $CovR_{T65}$ contributes to regulating CovR expression (16). In line with the results shown in Fig. 1B, the wild-type A20 strain and $A20_{CovR(T65A)}$ mutant had similar levels of *rgg* transcription (Fig. 3B). Nonetheless, the transcription of *rgg* was still suppressed in the $A20_{CovR(D53A-T65A)}$ mutant (Fig. 3B). In addition, the expression of SpeB was inhibited both in the transcriptional and translational levels in $A20_{CovR(D53A-T65A)}$ mutant compared to the wild-type A20 strain and $A20_{CovR(T65A)}$ mutant (Fig. 3C). These results indicate that phosphorylation at the T65 residue of CovR is not required for repression of *rgg* and *speB* transcription.

Expression of the $CovR_{D53A}$ protein in $CovR_{T65A}$ mutants inhibits *rgg* and *speB* transcription. Phosphorylated $CovR_{D53}$ has better DNA-binding activity than does the nonphosphorylated $CovR_{D53}$ protein (28); therefore, whether the nonphosphorylated $CovR_{D53}$ protein could inhibit *rgg* and *speB* transcription in the presence of phosphorylated $CovR_{D53}$ was further verified. The *covR* gene encoding the D53A amino acid substitution was carried by the pTRKL2 vector and was electroporated into the strain

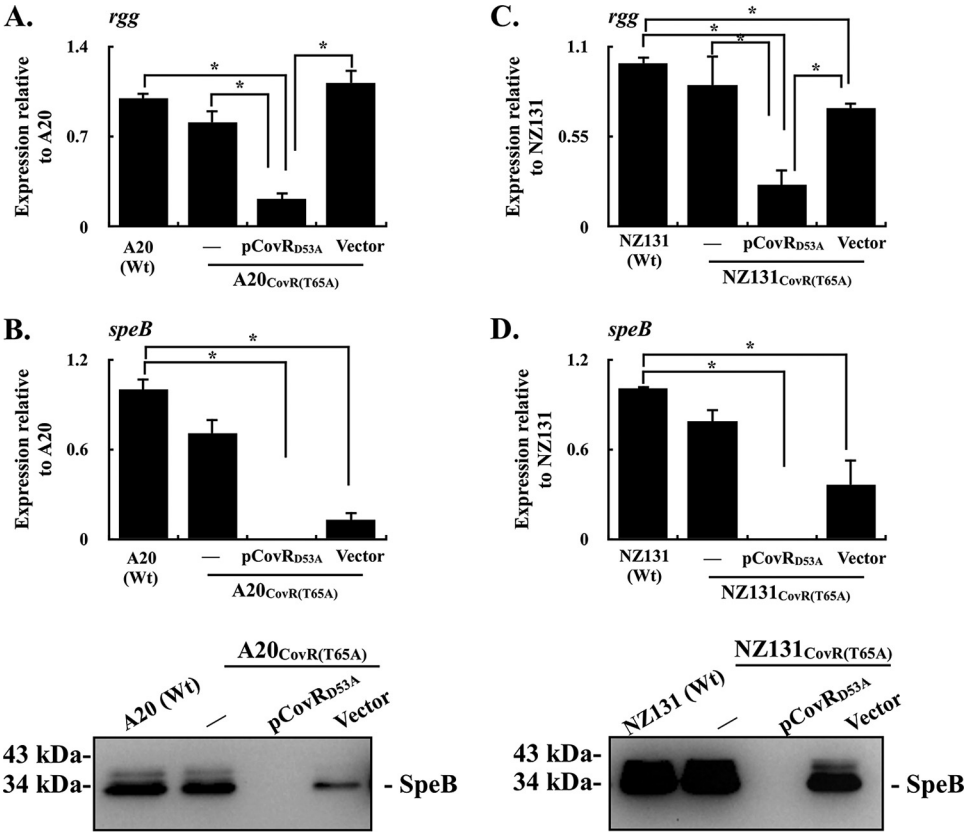

FIG 4 Expression of *rgg* and *speB* in CovR$_{T65A}$ mutants and their vector control and CovR$_{D53A}$ *trans*-complementary strains. Bacterial strains were cultured to the stationary phase of growth. RNAs and culture supernatants were collected for RT-qPCR and Western blot analyses. (A and B) Transcription of *rgg* and the transcriptional and translational levels of SpeB expression in the *emm*1-type A20 strain, CovR$_{T65A}$ mutant, and its vector control (Vector) and CovR$_{D53A}$ *trans*-complementary (pCovR$_{D53A}$) strains. (C and D) Transcription of *rgg* and the transcriptional and translational levels of SpeB expression in the *emm*49-type NZ131 strain, CovR$_{T65A}$ mutant, and its vector control and CovR$_{D53A}$ *trans*-complementary strains. Biological replicate experiments were performed using three independent preparations. The expression of *rgg* and *speB* was normalized to that of *gyrA*. Wt, wild-type strain; *, $P < 0.05$.

that expresses the phosphorylated CovR$_{D53}$ protein [A20$_{CovR(T65A)}$] (see Fig. S1 in the supplemental material). The transcription of *rgg* in the A20$_{CovR(T65A)}$ mutant, its vector control strain, and the CovR$_{D53A}$ *trans*-complemented strain were further compared. The results showed that *trans* complementation of the CovR$_{D53A}$ protein in the A20$_{CovR(T65A)}$ mutant suppressed the transcription of *rgg* compared to the wild-type A20 and its vector control strains (Fig. 4A). Next, the expression levels of SpeB in the wild-type A20 strain, A20$_{CovR(T65A)}$ mutant, its vector control strain, and the CovR$_{D53A}$ *trans*-complemented strain were analyzed by qPCR and Western blotting. Compared to the wild-type strain, the expression of SpeB in both transcriptional and translational levels was decreased in the vector control strain (Fig. 4B). Nonetheless, the expression of SpeB cannot be detected in the CovR$_{D53A}$ *trans*-complemented strain (Fig. 4B). Furthermore, the transcription of *rgg* and *speB* was similarly analyzed in NZ131 strains. In line with results shown in the A20 strains, the transcription of *rgg* and *speB* was inhibited in the CovR$_{D53A}$ *trans*-complemented strain of NZ131$_{CovR(T65A)}$ (Fig. 4C and D). These results suggest that the nonphosphorylated CovR$_{D53A}$ protein has a more dominant role in repressing the transcription of *rgg* and *speB* than does the phosphorylated CovR$_{D53}$ protein.

Expression of phosphorylated CovR$_{D53}$ protein in CovR$_{D53A}$ mutants cannot restore *rgg* and *speB* transcription. The transcription of *speB* is repressed in the *covS* mutant, suggesting that CovS acts as a positive regulator of *speB* (20, 31). In addition, it has been considered that CovS regulates downstream genes solely through CovR and

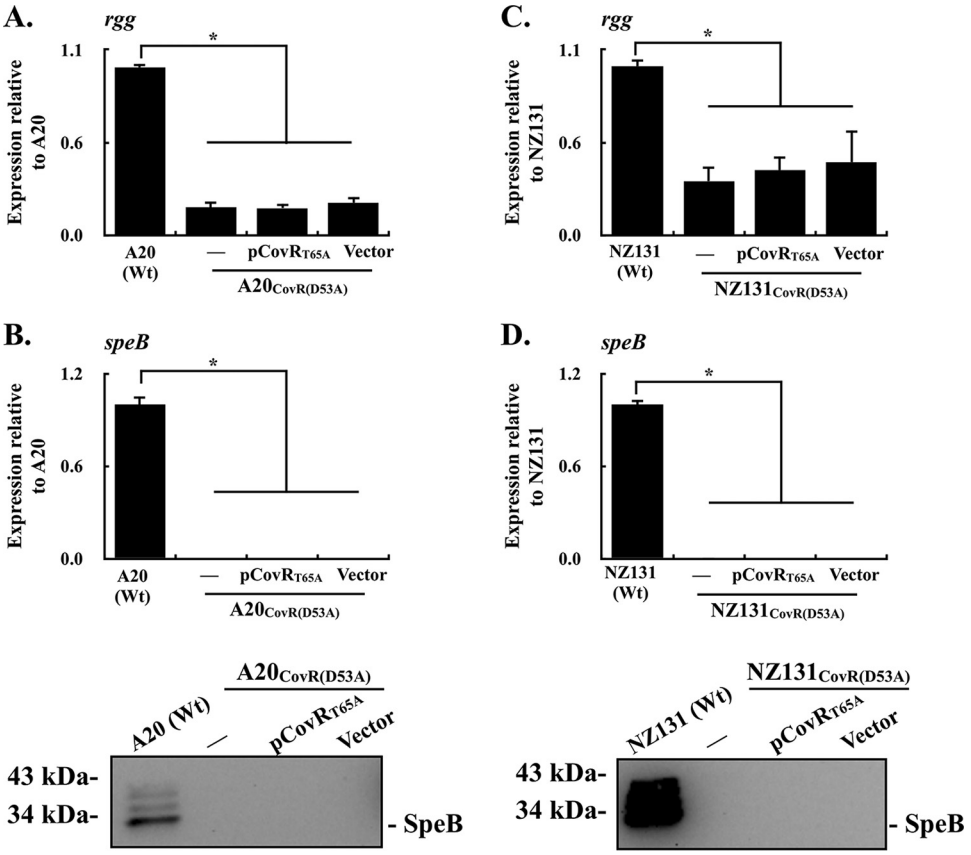

FIG 5 Expression of *rgg* and *speB* in CovR$_{D53A}$ mutants and their vector control and CovR$_{T65A}$ *trans*-complementary strains. Bacterial strains were cultured to the stationary phase of growth. RNAs and culture supernatants were collected for RT-qPCR and Western blot analyses. (A and B) Transcription of *rgg* and the transcriptional and translational levels of SpeB expression in the *emm*1-type A20 strain, CovR$_{D53A}$ mutant, and its vector control (Vector) and CovR$_{T65A}$ *trans*-complementary (pCovR$_{T65A}$) strains. (C and D) Transcription of *rgg* and the transcriptional and translational levels of SpeB expression in the *emm*49-type NZ131 strain, CovR$_{D53A}$ mutant, and its vector control and CovR$_{T65A}$ *trans*-complementary strains. Biological replicate experiments were performed using three independent preparations. The expression of *rgg* and *speB* was normalized to that of *gyrA*. Wt, wild-type strain; *, $P < 0.05$.

is required for the CovR phosphorylation (20). Therefore, whether the phosphorylated CovR$_{D53}$ could activate the *rgg* and *speB* transcription in A20$_{CovR(D53A)}$ was analyzed. The *covR* gene with the T65A amino acid substitution was carried by the pTRKL2 vector and was electroporated into CovR$_{D53A}$ mutants. The D53 residue of the CovR$_{T65A}$ protein can be phosphorylated by CovS, and expression of the phosphorylated CovR$_{T65A}$ protein in CovR$_{D53A}$ mutants was confirmed by Phos-tag Western blotting (Fig. 1; Fig. S1). Next, the transcription of *rgg* in the A20$_{CovR(D53A)}$ mutant, its vector-control strain, and the CovR$_{T65A}$ *trans*-complemented strain were further compared. The results showed that expression of the CovR$_{T65A}$ protein in the A20$_{CovR(D53A)}$ mutant had no effect on activating *rgg* and *speB* transcription (Fig. 5A and B). In addition, expression of the SpeB protein can only be detected in the wild-type A20 strain but not in A20$_{CovR(D53A)}$ and CovR$_{T65A}$ *trans*-complementary mutant strains (Fig. 5B). Furthermore, similar results were observed in NZ131 strains (Fig. 5C and D). These results indicate that the phosphorylated CovR$_{D53}$ cannot attenuate the repressor activity of the CovR$_{D53A}$ protein to *rgg* and *speB* transcription.

DISCUSSION

The phosphorylated CovR$_{D53}$ protein has better activity for binding to the *rgg-speB* intergenic region than does nonphosphorylated CovR$_{D53}$ (28). In addition, the transcription of *rgg* and *speB* is upregulated in the *covR* mutant, suggesting that CovR

inhibits *rgg* and *speB* transcription. Nonetheless, the transcription of *speB* is repressed in the *covS* mutant. In the present study, how phosphorylated and nonphosphorylated $CovR_{D53}$ proteins contribute to regulating *rgg* and *speB* expression was elucidated. The results showed that the nonphosphorylated $CovR_{D53}$ protein has a more dominant role in suppressing *rgg* and *speB* transcription than does the phosphorylated $CovR_{D53}$ protein. Furthermore, inactivation of the phosphorylation at D53 but not the T65 residue of CovR is crucial for repressing *rgg* and *speB* transcription.

The D53 ($CovR_{D53}$) and T65 ($CovR_{T65}$) residues of CovR can be phosphorylated, and the phosphorylation at these two residues is mutually exclusive (16). $CovR_{D53}$ is phosphorylated by CovS; however, $CovR_{T65}$ is phosphorylated by the serine/threonine kinase (16). The strain unable to be phosphorylated at $CovR_{T65}$ was hypervirulent compared to the wild-type strain (16), suggesting that the phosphorylated $CovR_{T65}$ could have an important role in modulating the regulatory activity of CovR. The present study showed that the transcription of *rgg* and *speB* was repressed in strains unable to be phosphorylated at $CovR_{D53}$ ($CovR_{D53A}$ mutants) but restored to levels similar to the wild-type strains in $CovR_{T65A}$ mutants (Fig. 1B and C and 2B and C), suggesting that phosphorylation at $CovR_{T65}$ could contribute to the repression of *rgg* and *speB* transcription. Nonetheless, inactivation of $CovR_{T65}$ phosphorylation in the $CovR_{D53A}$ mutant cannot derepress *rgg* and *speB* transcription (Fig. 3B and C). Although these results indicate that phosphorylation at the $CovR_{T65}$ is not required for inhibiting *rgg* and *speB* transcription, whether the phosphorylated $CovR_{T65}$ contributes to regulating other regulatory systems still needed to be verified.

The transcription of *covR* is negatively regulated by the CovR protein (28, 32). Compared to the wild-type A20 strain, the transcription of *covR* increased 2.95-fold $\pm$ 0.23-fold in its $CovR_{D53A}$ mutant. Nonetheless, the decreased expression of the CovR protein was observed in the $CovR_{D53A}$ mutants compared to that in the wild-type A20 and NZ131 strains (Fig. 1A and 2A). In addition, we also found that the expression level of CovR protein in the $CovR_{D53A}$ mutants was lower than that in the $CovR_{T65A}$ mutants (Fig. 1A and 2A). The underlying mechanisms of the decreased CovR protein in the $CovR_{D53A}$ mutants remain to be further studied; however, our results showed that the transcription of *rgg* and *speB* was not repressed in the $CovR_{T65A}$ mutants even though those mutants had higher levels of CovR protein than the $CovR_{D53A}$ mutants (Fig. 1 and 2). In addition, the present study showed that *trans* complementation of nonphosphorylated $CovR_{D53}$ in strains that express phosphorylated $CovR_{D53}$ resulted in the repression of *rgg* and *speB* transcription (Fig. 4). These results support the idea that the phosphorylated $CovR_{D53}$ has minor effects on repressing *rgg* and *speB* transcription. Furthermore, it would be reasonable to suggest that the nonphosphorylated $CovR_{D53}$ inhibits *rgg* and *speB* transcription through interacting with unknown regulatory systems but not through direct binding to the *rgg-speB* intergenic region.

In the *emm*3-type strain, the transcription levels of *speB* in *covR* deletion, $CovR_{D53A}$, and $CovR_{T65A}$ mutants were all upregulated compared to those of the wild-type strain (16). The present study showed that *trans* complementation of phosphorylated $CovR_{D53}$ in $CovR_{D53A}$ mutants cannot derepress *rgg* and *speB* transcription (Fig. 5), further supporting the notion that the CovR protein acts as the negative regulator for *rgg* and *speB*. Nonetheless, in the *emm*1- and *emm*49-type strains used in this study, the transcription of *speB* was repressed in the $CovR_{D53A}$ mutants but not in the $CovR_{T65A}$ mutants (Fig. 1C and 2C). In addition, *trans* complementation of the $CovR_{D53A}$ protein in strains that have the endogenous phosphorylated $CovR_{D53}$ protein resulted in the repression of *rgg* and *speB* transcription (Fig. 4). These results indicate that the nonphosphorylated $CovR_{D53}$ protein is crucial to mediate the repression of *rgg* and *speB* transcription. The different phenotypes observed in *emm*1-, *emm*49-, and *emm*3-type strains could be caused by the variations in the CovR/CovS phosphorelay signal among these strains (14). Nonetheless, how these variations influence the CovR/CovS regulatory activity to its target genes still needs to be further elucidated.

Rgg is required for triggering the expression of the cysteine protease SpeB (21, 33). In addition, inactivation of Rgg results in the increased resistance to oxidative stresses

TABLE 1 Plasmids and strains used in this study

Plasmid or strain	Parent strain	Description[a]	Reference or source
Plasmids			
pTRKL2		*E. coli-Streptococcus* shuttle vector	30
pCN143		Temperature-sensitive vector	30
pMW506		pSF152::*covR*Δ*cm*	30
pCN114		pSF152::*covS*Δ*cm*	This study
pCN129		pCN143::$CovR_{D53A}$	30
pCN160		pCN143::*covS*Δ*cm*	This study
pCN167		pCN143::$CovR_{T65A}$ (*covR* with A203G substitution)	This study
pCN169		pTRKL2::$CovR_{D53A}$	This study
pCN176		pCN143::$CovR_{D53A-T65A}$	This study
pCN180		pTRKL2::$CovR_{T65A}$	This study
Strains			
A20		*emm*1-type wild-type strain	40
NZ131		*emm*49-type wild-type strain	40
A20 Δ*covS*	A20	A20 *covS* isogenic mutant	This study
A20 Δ*covR*	A20	A20 *covR* isogenic mutant	30
NZ131 Δ*covS*	NZ131	NZ131 *covS* isogenic mutant	This study
NZ131 Δ*covR*	NZ131	NZ131 *covR* isogenic mutant	This study
$A20_{CovR(D53A)}$ mutant	A20	$CovR_{D53A}$ mutant strain	30
$NZ131_{CovR(D53A)}$ mutant	NZ131	$CovR_{D53A}$ mutant strain	This study
$A20_{CovR(T65A)}$ mutant	A20	$CovR_{T65A}$ mutant strain	This study
$NZ131_{CovR(T65A)}$ mutant	NZ131	$CovR_{T65A}$ mutant strain	This study
$A20_{CovR(D53A-T65A)}$ mutant	A20	$CovR_{D53A}$ and $CovR_{T65A}$ mutant strain	This study

[a]*cm*, chloramphenicol cassette.

as well as the overproduction of bacterial virulence factors, such as DNase and streptolysin O (SLO) (11, 23, 34); these phenotypes may lead to the increase in bacterial virulence. Spontaneous mutations in *rgg* were also frequently found in clinical isolates from patients with invasive manifestations (11). Nonetheless, Friães et al. (17) showed that acquisition of the *covS* null allele but not the *rgg* null allele is associated with invasive infections. The present study showed that nonphosphorylated $CovR_{D53}$ enhances its repression of *rgg* transcription (Fig. 1 to 4). These results suggest that inactivation of *covS* may not only derepress the expression of CovR-controlled genes but also upregulate the expression of Rgg-suppressed virulence genes. These phenomena may provide an explanation as to why the *covS* but not the *rgg* mutation is associated with invasive infections.

CovR/CovS is a two-component regulatory system; however, the *covS* and *covR* null mutants are not identical. The present study demonstrated that nonphosphorylated $CovR_{D53}$ has a more dominant role in repressing *rgg* and *speB* transcription than does the phosphorylated $CovR_{D53}$ protein. Whether the nonphosphorylated $CovR_{D53}$ represses *rgg* transcription directly or indirectly through binding to the *rgg-speB* intergenic region still needs to be verified; however, these results reveal the potential role of CovR in regulating its controlled genes in invasive *covS* mutants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. GAS A20 (*emm*1 type) and NZ131 (*emm*49 type) were described previously (30). GAS strains (Table 1) were cultured on Trypticase soy agar with 5% sheep blood or in tryptic soy broth (Becton, Dickinson and Company, Sparks, MD) supplemented with 0.5% yeast extract (TSBY). *Escherichia coli* DH5α was purchased from Yeastern Biotech Co., Ltd. (Taipei, Taiwan) and was cultured in Luria-Bertani (LB) broth at 37°C with vigorous aeration. When appropriate, the antibiotics chloramphenicol (25 μg/ml and 3 μg/ml for *E. coli* and GAS, respectively), spectinomycin (100 μg/ml), and erythromycin (150 μg/ml and 5 μg/ml for *E. coli* and GAS, respectively) were used for selection.

DNA and RNA manipulations. GAS genomic DNA extraction, RNA extraction, and reverse transcription were performed as described previously (35). Real-time PCR was performed in a 20-μl mixture containing 1 μl of cDNA, 0.8 μl of primers (10 μM), and 10 μl of SensiFAST SYBR Lo-ROX premixture (Bioline Ltd., London, UK), according to the manufacturer's instructions. Biological replicate experiments were performed from three independent RNA preparations in duplicate. The expression level of each target gene was normalized to that of *gyrA* and analyzed using the $\Delta\Delta C_T$ method (7500 software version 2.0.5; Applied Biosystems, Thermo Fisher Scientific, Inc.). In addition, all values of control and experi-

TABLE 2 Primers used in this study

Primer	Use	Sequence (5′ to 3′)[a]	Reference or source
covR-BamHI-F7	Construction	GCGGGATCCAAATGACAAAGAAAATTTTA	This study
covR-BamHI-R6	Construction	GCGGGATCCTAGGCACTTTCTTCTCAGAT	This study
covR-T65A-F	Construction	GATGGTTTTGAAGTGgCCCGTCGTTTGCAAACC	16
covR-T65A-R	Construction	GGTTTGCAAACGACGGGcCACTTCAAAACCATC	16
covS-F-2	Construction	CGGGATCCTCGTGAAGGGTTAGAAACTG	This study
covS-R-2-2	Construction	CCCCGGGCCATATGACTTATTTCTCACGAA	This study
covS-F-3-2	Construction	CCCCGGGGGCCCAGTCTAAAGAGAGTTAGAG	This study
covS-R-3-2	Construction	CCGGAATTCTTCGATTTCATCAACGTCCT	This study
Vec78_cat-F-1	Construction	CGCCGCGTTAACGATAGATTTATGATATAG	This study
Vec78_cat-R-1	Construction	CGCCGCGTTAACATTTATTCAGCAAGTCTT	This study
CovR/S-F-3	Construction	GCGGATCCGCTTGCAAGGGTTGTTTGATG	30
CovR-R-5	Construction	GCGGGATCCATGCAAGCCAGGAGATGATT	This study
Rgg-F-3	qPCR	TTTGAATGCCGAAACATAGAAGGTT	30
Rgg-R-2	qPCR	CTAATAACACCTTGACCAAGGCAAA	30
speB-F-2	qPCR	TGCCTACAACAGCACTTTGG	30
speB-R-2	qPCR	GGTAAAGTAGGCGGACATGC	30
gyrA-F-3	qPCR	CGTCGTTTGACTGGTTTGG	30
gyrA-R-3	qPCR	GGCGTGGGTTAGCGTATTTA	30

[a]Underlining indicates restriction enzyme sites; lowercase letters denote mutated nucleotides.

mental groups were divided by the mean of control samples before statistical analysis (36). Primers used for real-time PCR analysis (Table 2) were designed by Primer3 (version 0.4.0; http://frodo.wi.mit.edu) according to the MGAS5005 sequence (NCBI RefSeq accession no. NC_007297.1).

Construction of *covR* deletion, *covS* deletion, $CovR_{D53A}$, $CovR_{T65A}$, and $CovR_{D53A-T65A}$ mutants. The *covR* gene of NZ131 was interrupted by pMW506 with a double-cross homologous recombination, as described previously (30). To construct the *covS* isogenic mutants, the 592-bp *covS* upstream region and 659-bp *covS* downstream region were amplified with primers covS-F-2, covS-R-2-2, covS-F-3-2, and covS-R-3-2 and ligated into the vector pSF152 (30) to generate pCN114 (Tables 1 and 2). The chloramphenicol cassette of vector 78 (37) was amplified using primers Vec78_cat-F-1 and Vec78_cat-R-1 and ligated into pCN114 with the SmaI site. The upstream and downstream regions of *covS* and the chloramphenicol cassette in pSF152 were amplified by primers covS-F-2 and covR-3-2 and ligated into TA Cloning vector (Yeastern Biotech Co., Ltd., Taipei, Taiwan) and then transferred to the temperature-sensitive vector pCN143 (30) with the BamHI site (pCN160, Table 1). The amino acid residue substitution of CovR protein was generated by overlap PCR, as described previously (30). The T65A amino acid substitution of CovR was generated by primers CovR-BamHI-F7, CovR-T65A-R, CovR-BamHI-R6, and CovR-T65A-F from the wild-type A20 strain and its $CovR_{D53A}$ mutant. PCR products were ligated into the TA Cloning vector (Yeastern Biotech Co., Ltd.), and the amino acid substitution was confirmed by Sanger sequencing. The mutated *covR* genes were transferred to pCN143 with the BamHI site. The constructed plasmids (pCN160, pCN167, and pCN176) were transfected into strain A20 or NZ131 by electroporation, as described previously (38), and cultured at 30°C with the spectinomycin selection. Transformants were then transferred to 37°C to force the plasmid integration. Finally, transformants in which plasmid excised from the chromosome via recombination were selected by chloramphenicol (for *covS* and *covR* mutants) or in the blood agar plate (for $CovR_{D53A}$, $CovR_{T65A}$, and $CovR_{D53A-T65A}$ mutants) at 30°C. The *covS* and *covR* deletion mutants and GAS strains with the D53A substitution or D53A and T65A substitutions of CovR were confirmed by Sanger sequencing. All primers used are shown in Table 2.

Construction of vector control and *covR trans*-complemented strains. The *E. coli-Streptococcus* shuttle vector pTRKL2 was described previously (30). To construct *covR trans*-complemented strains, the genomic DNAs extracted from A20 $CovR_{D53A}$ and $CovR_{T65A}$ mutants served as the templates, and the *covR* gene, including its native promoter (1.3 kb), was amplified with primers CovR/S-F-3 and CovR-R-5 (Table 2). The PCR products were ligated into the BamHI site of pTRKL2, and constructed plasmids (pCN169 and pCN180) were transformed into $CovR_{T65A}$ or $CovR_{D53A}$ mutants with electroporation, as described previously (39).

Western blotting for SpeB and CovR proteins. Bacteria were cultured in TSBY broth for 8 h, and the culture supernatant was collected and sterilized with a 0.22-μm-pore-size filter for detecting SpeB protein. Thirty microliters of bacterial culture supernatants was mixed with 6× protein loading dye and separated by 12% SDS-PAGE gels. In order to analyze the expression of CovR protein, bacteria that were grown to exponential phase (6 h of incubation) were collected, and total proteins were extracted by the bead beater (Mini-beadbeater; BioSpec Products, Inc., Bartlesville, OK) (30). Ten micrograms of total protein was subjected to analysis with 12% SDS-PAGE gels. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA), and membranes were blocked by 5% skim milk in PBST buffer (PBS containing 0.2% Tween 20) at 37°C for 1 h. SpeB and CovR proteins were detected by the anti-SpeB antibody (Toxin Technology, Inc., Sarasota, FL) and anti-CovR serum (30), respectively. After hybridization, the membrane was washed with PBST buffer and hybridized with a secondary antibody, the peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution; Cell Signaling Technology, Inc., Danvers, MA), at room temperature for 1 h. The blot was developed using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Inc., Rockford, IL), and the signal was detected using the Gel Doc XR+ system (Bio-Rad).

Phos-tag Western blotting. Bacterial protein was extracted according to a previously described method (39). Ten micrograms of bacterial proteins was mixed with 6× protein loading dye (without boiling) and separated by 10% SDS-PAGE gels containing 10 μM Phos-tag (Wako Pure Chemical Industries Ltd., Richmond, VA) and 0.5 μM $MnCl_2$. Phosphorylated and nonphosphorylated proteins were separated on Phos-tag SDS-PAGE gels for 120 to 140 min at 100 V at 4°C. Protein transfer, membrane blocking, hybridization, and signal detection were performed as described previously (39).

Statistical analysis. Statistical analysis was performed by using the Prism software, version 5 (GraphPad, San Diego, CA). Significant differences in multiple groups were determined using analysis of variance (ANOVA). Posttest for ANOVA was analyzed by Tukey's honestly significant difference test. A *P* value of <0.05 was taken as significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00681-18.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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REFERENCES

1. Seale AC, Davies MR, Anampiu K, Morpeth SC, Nyongesa S, Mwarumba S, Smeesters PR, Efstratiou A, Karugutu R, Mturi N, Williams TN, Scott JA, Kariuki S, Dougan G, Berkley JA. 2016. Invasive group A *Streptococcus* infection among children, rural Kenya. Emerg Infect Dis 22:224–232. https://doi.org/10.3201/eid2202.151358.
2. Engelthaler DM, Valentine M, Bowers J, Pistole J, Driebe EM, Terriquez J, Nienstadt L, Carroll M, Schumacher M, Ormsby ME, Brady S, Livar E, Yazzie D, Waddell V, Peoples M, Komatsu K, Keim P. 2016. Hypervirulent *emm*59 clone in invasive group A *Streptococcus* outbreak, southwestern United States. Emerg Infect Dis 22:734–738. https://doi.org/10.3201/eid2204.151582.
3. Athey TB, Teatero S, Sieswerda LE, Gubbay JB, Marchand-Austin A, Li A, Wasserscheid J, Dewar K, McGeer A, Williams D, Fittipaldi N. 2016. High incidence of invasive group A *Streptococcus* disease caused by strains of uncommon *emm* types in Thunder Bay, Ontario, Canada. J Clin Microbiol 54:83–92. https://doi.org/10.1128/JCM.02201-15.
4. Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. Lancet Infect Dis 5:685–694. https://doi.org/10.1016/S1473-3099(05)70267-X.
5. Tse H, Bao JY, Davies MR, Maamary P, Tsoi HW, Tong AH, Ho TC, Lin CH, Gillen CM, Barnett TC, Chen JH, Lee M, Yam WC, Wong CK, Ong CL, Chan YW, Wu CW, Ng T, Lim WW, Tsang TH, Tse CW, Dougan G, Walker MJ, Lok S, Yuen KY. 2012. Molecular characterization of the 2011 Hong Kong scarlet fever outbreak. J Infect Dis 206:341–351. https://doi.org/10.1093/infdis/jis362.
6. Luk EY, Lo JY, Li AZ, Lau MC, Cheung TK, Wong AY, Wong MM, Wong CW, Chuang SK, Tsang T. 2012. Scarlet fever epidemic, Hong Kong, 2011. Emerg Infect Dis 18:1658–1661. https://doi.org/10.3201/eid1810.111900.
7. Hughes GJ, Van Hoek AJ, Sriskandan S, Lamagni TL. 2015. The cost of hospital care for management of invasive group A streptococcal infections in England. Epidemiol Infect 143:1719–1730. https://doi.org/10.1017/S0950268814002489.
8. Stevens DL. 2000. Streptococcal toxic shock syndrome associated with necrotizing fasciitis. Annu Rev Med 51:271–288. https://doi.org/10.1146/annurev.med.51.1.271.
9. Lin JN, Chang LL, Lai CH, Lin HH, Chen YH. 2014. Association between polymorphisms in the *csrRS* two-component regulatory system and invasive group A streptococcal infection. Eur J Clin Microbiol Infect Dis 33:735–743. https://doi.org/10.1007/s10096-013-2005-7.
10. Flores AR, Sahasrabhojane P, Saldana M, Galloway-Pena J, Olsen RJ, Musser JM, Shelburne SA. 2014. Molecular characterization of an invasive phenotype of group A *Streptococcus* arising during human infection using whole genome sequencing of multiple isolates from the same patient. J Infect Dis 209:1520–1523. https://doi.org/10.1093/infdis/jit674.
11. Ikebe T, Ato M, Matsumura T, Hasegawa H, Sata T, Kobayashi K, Watanabe H. 2010. Highly frequent mutations in negative regulators of multiple virulence genes in group A streptococcal toxic shock syndrome isolates. PLoS Pathog 6:e1000832. https://doi.org/10.1371/journal.ppat.1000832.
12. Ato M, Ikebe T, Kawabata H, Takemori T, Watanabe H. 2008. Incompetence of neutrophils to invasive group A *Streptococcus* is attributed to induction of plural virulence factors by dysfunction of a regulator. PLoS One 3:e3455. https://doi.org/10.1371/journal.pone.0003455.
13. Levin JC, Wessels MR. 1998. Identification of *csrR*/*csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A *Streptococcus*. Mol Microbiol 30:209–219. https://doi.org/10.1046/j.1365-2958.1998.01057.x.
14. Horstmann N, Sahasrabhojane P, Saldana M, Ajami NJ, Flores AR, Sumby P, Liu CG, Yao H, Su X, Thompson E, Shelburne SA. 2015. Characterization of the effect of the histidine kinase CovS on response regulator phosphorylation in group A *Streptococcus*. Infect Immun 83:1068–1077. https://doi.org/10.1128/IAI.02659-14.
15. Tran-Winkler HJ, Love JF, Gryllos I, Wessels MR. 2011. Signal transduction through CsrRS confers an invasive phenotype in group A *Streptococcus*. PLoS Pathog 7:e1002361. https://doi.org/10.1371/journal.ppat.1002361.
16. Horstmann N, Saldana M, Sahasrabhojane P, Yao H, Su X, Thompson E, Koller A, Shelburne SA. 2014. Dual-site phosphorylation of the control of virulence regulator impacts group A streptococcal global gene expression and pathogenesis. PLoS Pathog 10:e1004088. https://doi.org/10.1371/journal.ppat.1004088.
17. Friães A, Pato C, Melo-Cristino J, Ramirez M. 2015. Consequences of the variability of the CovRS and RopB regulators among *Streptococcus pyogenes* causing human infections. Sci Rep 5:12057. https://doi.org/10.1038/srep12057.
18. Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, Simpson AJ, Buchanan JT, Chhatwal GS, Kotb M, Nizet V. 2007. DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nat Med 13:981–985. https://doi.org/10.1038/nm1612.
19. Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM. 2006. Genome-wide analysis of group A streptococci reveals a mutation that modulates

global phenotype and disease specificity. PLoS Pathog 2:e5. https://doi.org/10.1371/journal.ppat.0020005.
20. Treviño J, Perez N, Ramirez-Pena E, Liu Z, Shelburne SA, Musser JM, Sumby P. 2009. CovS simultaneously activates and inhibits the CovR-mediated repression of distinct subsets of group A *Streptococcus* virulence factor-encoding genes. Infect Immun 77:3141–3149. https://doi.org/10.1128/IAI.01560-08.
21. Neely MN, Lyon WR, Runft DL, Caparon M. 2003. Role of RopB in growth phase expression of the SpeB cysteine protease of *Streptococcus pyogenes*. J Bacteriol 185:5166–5174. https://doi.org/10.1128/JB.185.17.5166-5174.2003.
22. Chaussee MS, Watson RO, Smoot JC, Musser JM. 2001. Identification of Rgg-regulated exoproteins of *Streptococcus pyogenes*. Infect Immun 69:822–831. https://doi.org/10.1128/IAI.69.2.822-831.2001.
23. Pulliainen AT, Hytonen J, Haataja S, Finne J. 2008. Deficiency of the Rgg regulator promotes H_2O_2 resistance, AhpCF-mediated H_2O_2 decomposition, and virulence in *Streptococcus pyogenes*. J Bacteriol 190:3225–3235. https://doi.org/10.1128/JB.01843-07.
24. Chaussee MA, Callegari EA, Chaussee MS. 2004. Rgg regulates growth phase-dependent expression of proteins associated with secondary metabolism and stress in *Streptococcus pyogenes*. J Bacteriol 186:7091–7099. https://doi.org/10.1128/JB.186.21.7091-7099.2004.
25. Chaussee MS, Somerville GA, Reitzer L, Musser JM. 2003. Rgg coordinates virulence factor synthesis and metabolism in *Streptococcus pyogenes*. J Bacteriol 185:6016–6024. https://doi.org/10.1128/JB.185.20.6016-6024.2003.
26. Federle MJ, Scott JR. 2002. Identification of binding sites for the group A streptococcal global regulator CovR. Mol Microbiol 43:1161–1172. https://doi.org/10.1046/j.1365-2958.2002.02810.x.
27. Gao J, Gusa AA, Scott JR, Churchward G. 2005. Binding of the global response regulator protein CovR to the *sag* promoter of *Streptococcus pyogenes* reveals a new mode of CovR-DNA interaction. J Biol Chem 280:38948–38956. https://doi.org/10.1074/jbc.M506121200.
28. Miller AA, Engleberg NC, DiRita VJ. 2001. Repression of virulence genes by phosphorylation-dependent oligomerization of CsrR at target promoters in *S. pyogenes*. Mol Microbiol 40:976–990. https://doi.org/10.1046/j.1365-2958.2001.02441.x.
29. Heath A, DiRita VJ, Barg NL, Engleberg NC. 1999. A two-component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect Immun 67:5298–5305.
30. Chiang-Ni C, Chu TP, Wu JJ, Chiu CH. 2016. Repression of Rgg but not upregulation of LacD.1 in *emm*1-type *covS* mutant mediates the SpeB repression in group A *Streptococcus*. Front Microbiol 7:1935. https://doi.org/10.3389/fmicb.2016.01935.
31. Tatsuno I, Okada R, Zhang Y, Isaka M, Hasegawa T. 2013. Partial loss of CovS function in *Streptococcus pyogenes* causes severe invasive disease. BMC Res Notes 6:126. https://doi.org/10.1186/1756-0500-6-126.
32. Gusa AA, Scott JR. 2005. The CovR response regulator of group A *Streptococcus* (GAS) acts directly to repress its own promoter. Mol Microbiol 56:1195–1207. https://doi.org/10.1111/j.1365-2958.2005.04623.x.
33. Lyon WR, Gibson CM, Caparon MG. 1998. A role for trigger factor and an *rgg*-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*. EMBO J 17:6263–6275. https://doi.org/10.1093/emboj/17.21.6263.
34. Anbalagan S, Chaussee MS. 2013. Transcriptional regulation of a bacteriophage encoded extracellular DNase (Spd-3) by Rgg in *Streptococcus pyogenes*. PLoS One 8:e61312. https://doi.org/10.1371/journal.pone.0061312.
35. Wang CH, Chiang-Ni C, Kuo HT, Zheng PX, Tsou CC, Wang S, Tsai PJ, Chuang WJ, Lin YS, Liu CC, Wu JJ. 2013. Peroxide responsive regulator PerR of group A *Streptococcus* is required for the expression of phage-associated DNase Sda1 under oxidative stress. PLoS One 8:e81882. https://doi.org/10.1371/journal.pone.0081882.
36. Valcu M, Valcu CM. 2011. Data transformation practices in biomedical sciences. Nat Methods 8:104–105. https://doi.org/10.1038/nmeth0211-104.
37. Tsou CC, Chiang-Ni C, Lin YS, Chuang WJ, Lin MT, Liu CC, Wu JJ. 2010. Oxidative stress and metal ions regulate a ferritin-like gene, *dpr*, in *Streptococcus pyogenes*. Int J Med Microbiol 300:259–264. https://doi.org/10.1016/j.ijmm.2009.09.002.
38. Chiang-Ni C, Tsou CC, Lin YS, Chuang WJ, Lin MT, Liu CC, Wu JJ. 2008. The transcriptional terminator sequences downstream of the *covR* gene terminate *covR/S* operon transcription to generate *covR* monocistronic transcripts in *Streptococcus pyogenes*. Gene 427:99–103. https://doi.org/10.1016/j.gene.2008.08.025.
39. Chiang-Ni C, Tseng HC, Hung CH, Chiu CH. 2017. Acidic stress enhances CovR/S-dependent gene repression through activation of the *covR/S* promoter in *emm*1-type group A *Streptococcus*. Int J Med Microbiol 307:329–339. https://doi.org/10.1016/j.ijmm.2017.06.002.
40. Chiang-Ni C, Zheng PX, Ho YR, Wu HM, Chuang WJ, Lin YS, Lin MT, Liu CC, Wu JJ. 2009. *emm*1/sequence type 28 strains of group A streptococci that express *covR* at early stationary phase are associated with increased growth and earlier SpeB secretion. J Clin Microbiol 47:3161–3169. https://doi.org/10.1128/JCM.00202-09.