Effect of Phosphatase Activity of the Control of Virulence Sensor (CovS) on Clindamycin-Mediated Streptolysin O Production in Group A *Streptococcus*

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ABSTRACT Severe manifestations of group A *Streptococcus* (GAS) infections are associated with massive tissue destruction and high mortality. Clindamycin (CLI), a bacterial protein synthesis inhibitor, is recommended for treating patients with severe invasive GAS infection. Nonetheless, the subinhibitory concentration of CLI induces the production of GAS virulent exoproteins, such as streptolysin O (SLO) and NADase, which would enhance bacterial virulence and invasiveness. A better understanding of the molecular mechanism of how CLI triggers GAS virulence factor expression will be critical to develop appropriate therapeutic approaches. The present study shows that CLI activates SLO and NADase expressions in the *emm1*-type CLI-susceptible wild-type strain but not in *covS* or control of virulence sensor (CovS) phosphatase-inactivated mutants. Supplementation with Mg^{2+} , which is a CovS phosphatase inhibitor, inhibits the CLI-mediated SLO up-regulation in a dose-dependent manner in CLI-susceptible and CLI-resistant strains. These results not only reveal that the phosphorylation of response regulator CovR is essential for responding to CLI stimuli, but also suggest that inhibiting the phosphatase activity of CovS could be a potential strategy for the treatment of invasive GAS infection with CLI.

KEYWORDS CovR/CovS, clindamycin, SLO, NADase, group A *Streptococcus*

Streptococcus pyogenes (group A *Streptococcus* [GAS]) is one of the most common pathogens causing necrotizing fasciitis and toxic shock syndrome (1). Clindamycin (CLI) is an antibiotic that interferes with bacterial protein synthesis and is recommended as an adjunctive therapy in patients with severe GAS infection (2, 3). Nonetheless, CLI has been characterized as an external signal that stimulates GAS to express virulent exoproteins such as streptolysin O (SLO), NADase, and DNases (4–6).

SLO, NADase, and the DNase Sda1 are negatively regulated by the control of virulence response regulator and sensor (CovR/CovS) two-component regulatory system (7–11). The sensor protein CovS, which has both kinase and phosphatase activities, modulates the phosphorylation levels of intracellular CovR (10, 12). The phosphorylated CovR has a better DNA-binding activity than nonphosphorylated CovR and is more effective at repressing the expressions of CovR-controlled genes (13, 14). CLI treatments have been shown to enhance the expression of SLO, NADase, DNases, and hyaluronic acid capsule (4–6). In addition, the expression of these virulence factors was not affected in *covS* mutants under CLI treatments (4). CovS is considered to regulate target genes solely through CovR (11). Nonetheless, the mechanism of how CovS modulates

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the phosphorylation levels of CovR in response to CLI treatments has not been clearly defined.

CovS modulates the phosphorylation on the D53 residue of CovR (12). The H280 and T284 residues are conserved in CovS, and mutations at the H280 and T284 residues abolish CovS's kinase and phosphatase activities, respectively (10, 12). Although the kinase activity of CovS is required for the optimal phosphorylation of CovR, it has been proposed that the levels of CovR phosphorylation are regulated by the phosphatase activity of CovS (12).

A better understanding of how CLI influences the CovR/CovS regulatory activity would be helpful for proposing appropriate approaches to treat patients with invasive GAS infections. The present study shows that the *covS* and CovR_{D53A} mutants cannot respond to CLI treatments to upregulate the SLO expression. In addition, inactivation of the phosphatase activity of CovS by genetic manipulation and supplementation with Mg²⁺ both impaired the CLI-mediated SLO upregulation. These results suggest that CLI inactivates CovS phosphatase activity to induce the expressions of CovR-controlled virulence exoproteins.

RESULTS

Clindamycin treatments induce SLO and NADase production in the wild-type strain but not in *covS* mutants. The phosphorylation level of CovR is modulated by CovS through its phosphatase activity (12). The *covS* and CovS phosphatase-inactivated (CovS_{T284A}) mutants were utilized to elucidate how CovR and CovS respond to clindamycin (CLI) treatments. The levels of phosphorylated CovR in the wild-type A20 strain, its *covS* mutant, and the CovS_{T284A} mutant were first analyzed using Phos-tag Western blotting. As expected, the phosphorylated CovR was detected in the A20 and CovS_{T284A} mutants but not in the *covS* mutant (Fig. 1A). In addition, a slight increase of phosphorylated CovR was observed in the CovS_{T284A} mutant compared to that of the wild-type A20 strain (Fig. 1A). The expression of GAS virulence factors is controlled by the growth-phase-dependent mechanisms (15); therefore, the growth activities of the CLI-susceptible wild-type A20 strain, its *covS* mutant, and the CovS_{T284A} mutant under different concentrations of CLI were measured. The results showed that the bacterial growth activity was significantly suppressed with treatment of 0.0375 μ g/ml of CLI (Fig. 1B) and therefore would result in the reduced NADase activity and SLO amount in the culture supernatants collected in the late-exponential phase of growth (Fig. 1C and D). At 0.01875 μ g/ml of CLI, the bacterial growth activity was moderately reduced (A20) or unaffected (*covS* and CovS_{T284A} mutants) compared to no-treatment conditions (Fig. 1B). Even though the growth activity was reduced, in the wild-type A20 strain, the NADase activity and SLO amount in the culture supernatants were significantly increased in the CLI-treated conditions compared to the no-treatment conditions in the stationary phase of growth (Fig. 1C and D). In the *covS* and CovS_{T284A} mutants, the NADase activity and SLO expression were not affected by the supplementation with 0.01875 μ g/ml of CLI during incubation (Fig. 1C and D). Again, the decreased NADase activity and SLO amount of the culture supernatant in the *covS* mutant were related to its attenuated growth activity under high CLI concentration (Fig. 1C and D). In addition, the NADase activity and SLO expression were further repressed in the CovS_{T284A} mutant compared to that in the wild-type A20 strain (Fig. 1C and D). These results not only demonstrate that CLI induces the expressions of CovR-controlled virulent exoproteins through CovS, but also reveal that the phosphatase activity of CovS would be required to respond to CLI stimuli.

Clindamycin treatments influence the levels of phosphorylated CovR and transcription of CovR-controlled *hasA* gene in the wild-type strain but not in the CovS phosphatase-inactivated mutant. The results shown in Fig. 1 suggest that CovS was required to respond to CLI treatments; therefore, whether CLI influences the phosphorylation levels of CovR in the wild-type A20 strain and CovS_{T284A} mutant was elucidated using Phos-tag Western blotting. Compared to the no-treatment conditions, the levels of phosphorylated CovR were decreased in the wild-type A20 strain but not

FIG 1 The phosphorylation levels of CovR, growth activity, NADase activity, and SLO expression in the wild-type A20 strain and its *covS* mutants under clindamycin (CLI)-treated and no-treatment conditions. (A) The phosphorylation levels of CovR in the wild-type A20 strain, its *covS* mutant ($\Delta covS$), *CovS* phosphatase-inactivated ($CovS_{T284A}$) mutant, and *covR* mutant ($\Delta covR$). GAS strains were cultured to the late-exponential phase of growth, and total proteins were extracted for Phos-tag Western blot analysis. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. The total protein was used as the loading control. (B) The growth activity of A20, *covS* mutant, and $CovS_{T284A}$ mutant with 0, 0.01875, and 0.0375 $\mu\text{g/mL}$ of CLI. The overnight-cultured bacteria were transferred (1:100) to TSBY broth supplementation with or without CLI, and the turbidity of bacterial suspension was measured at an absorbance of 600 nm (OD₆₀₀). *, $P < 0.05$. (C) NADase activity and (D) expression of SLO in the wild-type A20 strain, *covS* mutant, and $CovS_{T284A}$ mutant with 0, 0.01875, and 0.0375 $\mu\text{g/mL}$ of CLI. The GAS strains were cultured to the late exponential and stationary phases, and bacterial culture supernatants were collected for determining NADase activity and Western blot analysis.

in the $CovS_{T284A}$ mutant in the late exponential phase of growth under 0.0375 $\mu\text{g/mL}$ of CLI-treated conditions (Fig. 2A). Nonetheless, in the stationary phase of growth, the increase of phosphorylated CovR was observed in both A20 and $CovS_{T284A}$ mutants under CLI-treated conditions (Fig. 2A). In addition, the Western blot analysis showed that there were no dramatic changes in the expression levels of CovR in the A20 and $CovS_{T284A}$ mutant strains under the CLI-treated and no-treatment conditions (Fig. 2A). These results suggest that the phosphatase activity of CovS could be activated by CLI to reduce levels of phosphorylated CovR in the late exponential phase of growth. In the stationary phase of growth, CLI may play a minor role in affecting CovS phosphatase activity.

CLI treatments resulted in reduced levels of phosphorylated CovR in the late-exponential phase of growth (Fig. 2A); however, the difference was small. In order to demonstrate that the CLI treatments influenced the phosphorylation of CovR and expression of SLO, similar analyses in another *emm1*-type CLI-susceptible SF370 strain were performed. Similar to the results shown in Fig. 2A, the levels of phosphorylated

FIG 2 The phosphorylation levels of CovR and expressions of CovR, SLO, and *hasA* transcript in the wild-type strains and CovS phosphatase-inactivated (CovS_{T284A}) mutant under clindamycin (CLI)-treated and no-treatment conditions. (A) The levels of expression and phosphorylation of CovR in the wild-type A20 strain and CovS_{T284A} mutant with 0, 0.01875, and 0.0375 μg/ml of CLI. (B) The phosphorylation levels of CovR and the expression of CovR and SLO in the CLI-susceptible *emm1*-type SF370 strain with 0, 0.01875, and 0.0375 μg/ml of CLI. GAS strains were cultured to the late-exponential and stationary phases, and bacterial cells were collected for Phos-tag Western blot (for detecting phosphorylated CovR protein) and Western blot (for detecting total CovR protein) analyses. The culture supernatants were utilized for detecting SLO expression via Western blotting. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. The *covR* mutant (Δ*covR*) and *covS* mutant (Δ*covS*) served as the experimental controls. The total protein was used as the loading control. (C) The transcription of *hasA* in the wild-type A20 strain and CovS_{T284A} mutant with 0 and 0.0375 μg/ml of CLI. GAS strains were cultured to the late exponential and stationary phases, and RNAs were collected for reverse transcription (RT)-qPCR analysis. Biological replicate experiments were performed using three independent preparations. The expression of *hasA* was normalized to that of *gyrA*. *, *P* < 0.05.

CovR in the SF370 strain were repressed under exposure to 0.0375 μg/ml of CLI in the late exponential phase (Fig. 2B). In the stationary phase of growth, the increase in phosphorylated CovR was observed under CLI-treated conditions (Fig. 2B). In addition, the expression of SLO was upregulated under CLI-treated conditions (Fig. 2B).

The phosphorylated CovR protein binds to the promoter of the *hasABC* operon (which is responsible for hyaluronic acid capsule synthesis) to inhibit its transcription (14, 16). In order to clarify whether CLI influences the phosphorylation levels of CovR, the transcription of *hasA* in the A20 and CovS_{T284A} mutant in the presence or absence of CLI was analyzed using quantitative PCR (qPCR). Compared with the no-treatment conditions, the transcription of *hasA* was increased in the A20 strain but similar in the CovS_{T284A} mutant under CLI-treated conditions (Fig. 2C), indicating that CovS's phosphatase activity was required to derepress the *hasA* transcription under CLI-treated conditions.

Phosphorylation levels of CovR and the expression of SLO in the CovS phosphatase trans-complementary strain under Mg²⁺ or clindamycin-treated conditions. To further clarify the role of CovS phosphatase activity in response to CLI treatments, the phosphatase activity of CovS was complemented in the CovS_{T284A} mutant, and the levels of phosphorylated CovR and expression of SLO under CLI-treated and no-treatment conditions were compared. The levels of phosphorylated

FIG 3 The phosphorylation levels of CovR and the expression of SLO in the wild-type A20 strain, the CovS_{T284A} mutant, its empty vector-controlled strain, and the CovS phosphatase complementary strain under CLI or Mg²⁺ treatments. GAS strains were cultured to the stationary phase of growth, and total bacterial proteins and culture supernatants were collected. Total proteins were utilized for Phos-tag Western blot (for detecting phosphorylated CovR protein) and Western blot (for detecting total CovR) analyses. The culture supernatants were utilized for detecting SLO expression via Western blotting. (A) The phosphorylation levels of CovR and the expression of SLO in the CovS_{T284A} mutant, its empty vector-controlled strain [CovS_{T284A}(Empty Vec)], and the CovS phosphatase complementary strain [CovS_{T284A}(pCovS_{H280A})] with 0, 0.01875, and 0.0375 μg/ml of CLI. (B) The phosphorylation levels of CovR and the expression of SLO in A20, the empty vector-controlled strain of the CovS_{T284A} mutant [CovS_{T284A}(Empty Vec)], and the CovS phosphatase complementary strain [CovS_{T284A}(pCovS_{H280A})] with CLI and/or Mg²⁺ stimuli. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. The total protein was used as the loading control.

CovR were slightly decreased in the *trans*-complementary strain compared to that in the empty vector-controlled strain (Fig. 3A); however, the difference was small. The expression of SLO was increased in this complementary strain compared to the CovS_{T284A} mutant and its empty vector-controlled strain (Fig. 3A), indicating that the phosphatase activity of CovS was required for derepressing SLO expression. Nonetheless, supplementation with CLI did not result in an upregulation of SLO expression, nor did it affect the levels of phosphorylated CovR in the complementary strain (Fig. 3A). Mg²⁺ has been shown as a signal to inactivate the phosphatase activity of CovS (12). Supplementation with Mg²⁺ during incubation repressed the expression of SLO in the wild-type A20 and CovS phosphatase *trans*-complementary strains compared to the untreated conditions (Fig. 3B), indicating that the complementation successfully restored the phosphatase activity of CovS in the CovS_{T284A} mutant. Nonetheless, the CLI treatments did not result in an upregulation of SLO expression in the complementary strain (Fig. 3B). Although the low-copy-number vector was utilized, the expression level of CovS in the complementary strain should be higher than that of the wild-type strain. Therefore, the effects of CLI treatments on SLO expression cannot be determined in this complementary strain.

Supplementation with Mg²⁺ represses the CLI-mediated SLO upregulation. Although the phosphatase activity of CovS is required for the upregulation of SLO

FIG 4 The levels of phosphorylated CovR and the expressions of CovR, SLO, and *slo* transcript in the wild-type A20 strain, its CovS phosphatase-inactivated mutant (CovS_{T284A}), and the strain that cannot phosphorylate D53 residue of CovR (CovR_{D53A}) under Mg²⁺ and clindamycin (CLI) supplementation conditions. (A) The expression of SLO in the CovR_{D53A} mutant under Mg²⁺ and CLI supplementation conditions. (B) The levels of phosphorylated CovR and expression of CovR and SLO proteins in the wild-type A20 strain in the Mg²⁺ and/or CLI supplementation conditions. Bacteria were cultured to the stationary phase of growth, and the total bacterial proteins and culture supernatants were collected. Total proteins were utilized for Phos-tag Western blot (for detecting phosphorylated CovR protein) and Western blot (for detecting total CovR) analyses. The culture supernatants were utilized for detecting SLO expression with Western blot. CovR~P, phosphorylated CovR; CovR, nonphosphorylated CovR. The *covS* mutant (Δ*covS*) served as the experimental control. Total protein serves as the loading control. (C) The transcription of *slo* in the A20 and CovS_{T284A} mutant under Mg²⁺ and/or CLI supplementation conditions. GAS strains in the late exponential phase were collected for RT-qPCR analysis. Biological replicate experiments were performed using three independent preparations. The expression of *slo* was normalized to that of *gyrA*. *, *P* < 0.05. (D) The expression of SLO in the wild-type A20 strain under Mg²⁺ and CLI supplementation conditions. Bacteria were cultured to the stationary phase of growth, and the bacterial culture supernatant was collected for detecting SLO using Western blotting.

under CLI treatments (Fig. 1D), the results shown in Fig. 3 did not fully support the hypothesis that CLI could activate CovS phosphatase activity. The phosphatase activity of CovS has been shown to be inhibited by Mg²⁺ (12). If CLI induces SLO production by activating the phosphatase activity of CovS, supplementation with Mg²⁺ should inactivate the CLI-mediated SLO upregulation. We first verified whether both CLI and Mg²⁺ regulated SLO expression by modulating the phosphorylation levels of CovR. The expression of SLO was analyzed under CLI and Mg²⁺ stimuli in the strain that cannot phosphorylate CovR (CovR_{D53A} mutant), and the results showed that the expression of SLO was not affected in the presence of Mg²⁺ or CLI (Fig. 4A).

Next, whether Mg²⁺ could be utilized as an antagonist to inactivate CLI-mediated SLO upregulation was analyzed. The results showed that Mg²⁺ supplementation significantly inhibited SLO production under CLI-free and CLI-treated conditions (Fig. 4B). Nonetheless, the Phos-tag Western blotting cannot be used to clearly demonstrate the changes in the phosphorylation level of CovR under the Mg²⁺ stimuli (Fig. 4B). In addition, the transcription of *slo* was analyzed using qPCR under similar conditions. As expected, the transcription of *slo* in the A20 strain was increased under CLI-treated conditions compared to that under no-treatment conditions (Fig. 4C). Supplementation with Mg²⁺ under CLI-free and CLI-treated conditions repressed the

FIG 5 The growth activity and SLO expression in the clindamycin (CLI) nonsusceptible *emm12*-type SPY128 and *emm73*-type SPY139 isolates under Mg²⁺ and CLI supplementation conditions. (A) The growth activities of the SPY128 and SPY139 isolates under different concentrations of CLI treatments. The overnight-cultured bacteria were transferred (1:100) to TSBY broth supplemented with or without CLI, and the turbidity of the bacterial suspension was measured at an absorbance of 600 nm (OD₆₀₀). *, *P* < 0.05. (B) The expression of SLO in the SPY128 and SPY139 isolates under different concentrations of CLI treatments. (C) The expression of SLO in the SPY128 and SPY139 isolates under Mg²⁺ and CLI supplementation conditions. GAS strains were cultured to the stationary phase, and culture supernatants were collected for Western blot analysis.

slo transcription in the wild-type A20 strain but not in the CovS_{T284A} mutant (Fig. 4C). Furthermore, in the CovS_{T284A} mutant, there was no difference in the *slo* transcription under the Mg²⁺ or CLI stimuli, indicating that CovS's phosphatase activity was required for regulating *slo* transcription in response to Mg²⁺ and CLI treatments. Finally, whether Mg²⁺ could inhibit CLI-mediated SLO upregulation in a dose-dependent manner was analyzed. Similar to the results shown in Fig. 4B, the expression of SLO in the A20 strain was increased in the presence of CLI treatments (Fig. 4D). The expression of SLO was not repressed in the presence of 1 mM of Mg²⁺ but was instead restored to levels similar to those in CLI-free conditions when 5 mM of Mg²⁺ was added (Fig. 4D). In addition, under the CLI-treated conditions, the SLO expression was repressed to levels lower than those in CLI-free conditions after supplementation with 10 and 15 mM of Mg²⁺ (Fig. 4D). These results indicated that the CLI-mediated SLO upregulation was inhibited by Mg²⁺ in a dose-dependent manner.

Supplementation with Mg²⁺ represses the CLI-mediated SLO upregulation in CLI-nonsusceptible *emm12*-type and *emm73*-type isolates. The CLI-mediated upregulation of SLO was further validated in the CLI-resistant SPY128 (*emm12*) and inducible CLI-resistant SPY139 (*emm73*) isolates. Supplementation with 0.01875 and 0.0375 μg/ml of CLI significantly induced SLO expression in the CLI-resistant SPY128 isolate (data not shown). Although high concentrations of CLI (1.5 μg/ml and 0.0375 μg/ml for the SPY128 and SPY139 isolates, respectively) attenuated the growth activities of SPY128 and SPY139 (Fig. 5A), the expression of SLO was increased under CLI-treated conditions compared to the no-treatment conditions (Fig. 5B). Furthermore, the expressions of SLO after supplementation with or without Mg²⁺ and CLI were analyzed using Western blotting. The results showed that supplementation with Mg²⁺ (5 to 15 mM) inhibited SLO expression under CLI-treated conditions in the SPY128 and SPY139 isolates (Fig. 5C). These results indicated that the supplementation with Mg²⁺ inhibited the CLI-mediated SLO upregulation of the CLI-nonsusceptible GAS isolates.

DISCUSSION

Clindamycin (CLI) has been utilized for treating patients with severe GAS infection. Nonetheless, in addition to the CLI-resistant GAS that has been reported (17–23), CLI has been shown to be an external signal of the CovR/CovS system to stimulate GAS to express SLO, NADase, and DNases (4–6). Although changes in the phosphorylation levels of CovR protein cannot be clearly demonstrated by Phos-tag Western blotting, our results indicated that the phosphatase activity of CovS was required for GAS to sense the CLI treatments to derepress the production of SLO and NADase in CLI-susceptible and nonsusceptible strains.

Andreoni et al. (6) showed that the subinhibitory concentration of CLI induces GAS to mirror the virulent animal-passaged (AP) phenotype. The AP phenotype, including upregulation of SLO and downregulation of the cysteine protease SpeB, has been found in strains that cannot phosphorylate CovR protein (7, 8). The present study indicated that CLI treatments would activate CovS phosphatase activity to upregulate SLO expression, further supporting the observations from Andreoni et al. (6). In addition, our previous study showed that the repression of *speB* is mediated by the nonphosphorylated CovR (24). Therefore, the decrease of SpeB production in the wild-type A20 strain under CLI-treated conditions (data not shown) could be mediated by an increase in the nonphosphorylated CovR protein. These results demonstrated that CLI influences the expression of SLO and SpeB by modulating the levels of intracellular phosphorylated CovR.

Nonetheless, the changes in the level of phosphorylated CovR under CLI treatments were not dramatic (Fig. 2 and 3). In addition, supplementation with Mg^{2+} , but not CLI, influenced the expression of SLO in the CovS phosphatase-complementary strain (Fig. 3). The promoter activity of the CovR-controlled *hasA* gene was reduced about 50% in the presence of 5 mM of Mg^{2+} compared to no-treatment conditions (25); however, the CLI-mediated SLO upregulation was significantly repressed after supplementation with 5 mM of Mg^{2+} (Fig. 4D and 5C). These results suggested that CLI was not a strong signal in the CovR/CovS system compared to Mg^{2+} . Furthermore, the sensitivity of the Phos-tag Western blot was not good enough to quantify the changes of phosphorylated proteins in bacteria. New analytical approaches are needed to reveal the phosphorelay pathway between sensor and response proteins in bacteria.

The results from this study showed that the decrease in phosphorylated CovR under CLI-treated conditions in the wild-type A20 strain was only observed in the exponential phase but not in the stationary phase (Fig. 2A). In line with these observations, Sawai et al. (5) indicated that SLO production was only increased when CLI was added into the culture before the mid-log phase. These results suggest that unknown environmental factors in the stationary phase- or growth phase-dependent regulatory mechanism would upregulate the phosphorylation level of CovR under CLI-treated conditions. It has been proposed that intracellularly persistent GAS remain in the late stationary phase because either the nutrient supply was limited or the environment in the phagosome was filled with growth-inhibitory substances (26). Therefore, these results may also provide hints as to why CLI was still effective in reducing tissue damage even when the *in vitro* analysis showed that the expressions of SLO and NADase were upregulated under CLI treatments (4, 6).

Horstmann et al. (12) showed that a high concentration of Mg^{2+} impaired the phosphatase activity of CovS and resulted in increased levels of phosphorylated CovR. In the present study, we found that the phosphatase activity of CovS was required for GAS to respond to CLI stimuli (Fig. 1D and 4C). In addition, the expression of SLO in clindamycin-susceptible and -nonsusceptible strains was effectively repressed by supplementation with Mg^{2+} under CLI-treated conditions (Fig. 4D and 5C). Furthermore, supplementation with Mg^{2+} had no effects on SLO expression in the A20 CovR_{D53A} mutant under CLI-treated conditions (Fig. 4A). The total Mg^{2+} concentration ranged from 17 to 20 mM in the majority of mammalian cell types examined. Cytoplasmic Mg^{2+} is around 4 to 5 mM and is present in the form of a complex with ATP,

TABLE 1 Plasmids and strains used in this study

Plasmid or strain	Description	Reference or source
Plasmids		
pCN143	Temperature-sensitive vector	30
pTRKL2	<i>E. coli</i> -GAS shuttle vector	30
pCN161	pCN143::CovR _{T284A}	This study
pCN205	pTRKL2::covS (with H280A substitution)	This study
Strains		
A20	<i>emm1</i> /ST28 wild-type strain	29
SF370	<i>emm1</i> /ST28 strain	
A20 Δ covS	A20 covS isogenic mutant	24
A20 Δ covR	A20 covR isogenic mutant	30
A20 CovR _{D53A}	A20 CovR _{D53A} mutant strain	30
A20 CovS _{T284A}	A20 CovS _{T284A} mutant strain	This study
SPY128	<i>emm12</i> type, clindamycin-resistant strain	This study
SPY139	<i>emm73</i> type, inducible clindamycin-resistant strain	This study

phosphonucleotides, and phosphometabolites (27). In the present study, we found that 5 mM of Mg²⁺ treatment could effectively repress the CLI-mediated SLO production (Fig. 4D and 5C). These results not only demonstrate that both Mg²⁺ and CLI regulated SLO expression through modulation of phosphatase activity of CovS, but also suggest that supplementation with a CovS phosphatase activator could attenuate the effects on the production of CLI-induced virulence factors. Nonetheless, Mg²⁺ is biologically important to both bacteria and eukaryotic cells. Therefore, more models based on cell or animal infections are needed to evaluate the effect of Mg²⁺ on CLI treatments of invasive GAS infections.

This study revealed the molecular mechanism of how CLI upregulated SLO expression through the CovR/CovS system. More importantly, these results suggested that the phosphatase activity of CovS is not only critical for the pathogenesis of GAS (28), but is also a promising target for the treatment of invasive GAS infection with CLI.

MATERIALS AND METHODS

Bacterial strains and culture conditions. GAS A20 and SF370 are *emm1*/sequence type 28 (ST28) strains that were described previously (29). SPY128 (*emm12* type) and SPY139 (*emm73* type) are clinical isolates provided by the Bacterial Bank of LinKou Chang Gung Memorial Hospital, Taiwan (Table 1). The susceptibility to clindamycin of GAS was determined with the disc diffusion and D test assays according to protocols described by the Clinical and Laboratory Standards Institute (33). GAS strains and *Escherichia coli* DH5 α (Yeastern Biotech Co., Ltd., Taipei, Taiwan) were cultured as described previously (24). When appropriate, the antibiotics spectinomycin (100 μ g/ml), erythromycin (5 μ g/ml and 125 μ g/ml for GAS and *E. coli*, respectively), and chloramphenicol (3 μ g/ml and 25 μ g/ml for GAS and *E. coli*, respectively) were used for selection.

DNA and RNA manipulations. GAS genomic DNA extraction, RNA extraction, and reverse transcription were performed as described previously (30). Biological replicate experiments were performed in duplicate from three independent RNA preparations. The expression level of each target gene was normalized to that of *gyrA* and analyzed using the $\Delta\Delta$ Ct method (7500 software v2.0.5, Applied Biosystems, Thermo Fisher Scientific, Inc.). In addition, all values of control and experimental groups were divided by the mean of control samples before statistical analysis (31). Primers used for real-time PCR analysis were designed using Primer3 v0.4.0 (<http://frodo.wi.mit.edu>) according to the MGAS5005 sequence (NCBI accession no. NC_007297.1) and are described in Table 2.

Construction of the CovS_{T284A} mutant and its CovS phosphatase trans-complementary strain. The T284A amino acid substitution in CovS was constructed using overlap PCR with primers CovS-BamHI-F6, CovS-BamHI-R6, CovS-T284A-F6, and CovS-T284A-R6 (Table 2) (12). The nucleotide sequence of the PCR product (780 bp) was confirmed with sequencing and ligated into the temperature-sensitive vector pCN143 (30). The constructed plasmid (pCN161, Table 1) was transfected into GAS with electroporation and cultured at 30°C. Transformants were transferred to 37°C to force the plasmid integration via a single homologous recombination. Finally, transformants in which the plasmid excised from the chromosome via a second recombination were selected in the antibiotic-free plate at 30°C. The transformants with the T284A mutation in the *covS* gene were verified with sequencing. To complement the phosphatase activity of CovS in the CovS_{T284A} mutant, the *covR/covS* genes with its native promoter were amplified from the CovS kinase-inactivated CovS_{H280A} mutant (24). The *covR* gene amplified from the CovS_{H280A} mutant was further removed using PCR with two reverse primers. The PCR product was ligated into the low-copy-number vector pTRKL2 (24) and transformed into the CovS_{T284A} mutant to complement the CovS phosphatase activity.

TABLE 2 Primers used in this study

Primer	Use	Sequence (5' to 3') ^a	Reference or source
hasA-F-2	qPCR	tgaagatctgacgctgacg	9
hasA-R-2	qPCR	accccaaggcattatcgta	9
slo-F-1	qPCR	gcagagcacaataaggtagt	9
slo-R-1	qPCR	ctggtgatgaaataggataag	9
gyrA-F-3	qPCR	cgctgttgactggtttgg	30
gyrA-R-3	qPCR	ggcgtgggttagcgtattta	30
CovS-BamHI-F6	Construction	gcgggatccagaatcatctcctgcttgc	12
CovS-BamHI-R6	Construction	gcgggatccatcaagcctgctcaaaa	12
CovS-T284A-F6	Construction	ccatgaattacgCGcaccggttgcgatta	12
CovS-T284A-R6	Construction	taatcgcaaccggtgCGcgaattcatgg	12
CovS-F-4	Construction	acatgcatgcatggaatcagaaacaaaa	This study
CovS-R-4	Construction	acatgcatgcttataccaacccttctc	This study

^aUnderlining indicates restriction enzyme site; uppercase letters denote mutated nucleotides.

Measurement of bacterial growth activity. GAS strains were cultured in Trypticase soy broth with yeast extract (TSBY broth) at 37°C for 12 to 16 h. The 100- μ l bacterial suspension was transferred to 10 ml of fresh TSBY broth containing different concentrations of clindamycin and cultured at 37°C and 5% CO₂. After 2, 4, 6, and 8 h of incubation, the turbidity of bacterial suspensions was determined at an absorbance of 600 nm (OD₆₀₀).

Measurement of NADase activity. The NADase activity of the bacterial culture supernatant was measured by the method described previously (9). Briefly, bacterial culture supernatants from different culture conditions were collected by centrifugation at 10,000 $\times g$ and sterilized using a 0.22- μ m filter (Merck Millipore, Billerica, MA). A total of 25 μ l of bacterium-free supernatant was mixed with 50 μ l of NADase substrate (1 mM of β -NAD; N8535, Sigma-Aldrich) in a 96-well plate and incubated at 37°C in 5% CO₂ in an incubator for 1 h. To develop reactions, 50 μ l of NaOH (2 N) was added, and the plate was incubated in the dark at room temperature for an additional hour. The supernatant that exhibited no fluorescence indicates that all β -NAD has been converted to nonfluorescence products by NADase (32).

Western blot analysis. Bacterial culture supernatants were collected using centrifugation (10,000 $\times g$ at 4°C for 10 min) and sterilized using a 0.22- μ m filter. The 30 μ l of bacterial culture supernatants was mixed with 4 $\times$ protein loading dye and separated using 12% SDS-PAGE. The gel was washed with transfer buffer (39 mM of glycine, 48 mM of Tris, 0.037% of SDS, and 20% methanol), and proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore). Membranes were incubated with 5% skim milk in phosphate-buffered saline with Tween 20 (PBST) buffer (PBS containing 0.2% Tween 20) at 37°C for 1 h, and CovR and SLO were detected using mouse anti-CovR serum (30) and anti-SLO antibody (Abcam, Cambridge, UK), respectively. After hybridization, primary antibody was washed from the membrane with PBST and hybridized with the secondary antibody peroxidase-conjugated rabbit anti-mouse/rabbit IgG (1:10,000 dilution; Merck Millipore) at room temperature for 1 h. The blot was developed using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Inc., Rockford, IL), and a signal was detected using the Gel Doc XR+ system (Bio-Rad, Hercules, CA).

Phos-tag Western blot analysis. Phos-tag Western blotting used to detect phosphorylated CovR was performed as described previously (30). Briefly, bacterial cells were disrupted by a bead beater, and 3 μ g of total protein was mixed with 4 $\times$ protein dye and subjected to 10% SDS-PAGE containing 10 μ M of Phos-tag (Wako Pure Chemical Industries Ltd., Richmond, VA) and 0.5 μ M of MnCl₂ without boiling. Phosphorylated proteins were separated on a Phos-tag SDS-PAGE system for 120 to 140 min at 100 V at 4°C. Protein transfer, membrane blocking, hybridization, and signal detection were performed as described previously (30).

Statistical analysis. Statistical analysis was performed by using Prism software v5 (GraphPad, San Diego, CA). Significant differences in multiple groups were determined using analysis of variance (ANOVA). Posttesting of ANOVA was analyzed using Tukey's honestly significant difference test. A *P* value of <0.05 was taken as significant.

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