The Two-Component Sensor Protein CovS Affects Penicillling Susceptibility by Modulation of Cell-Wall Synthesis in Streptococcus pyogenes

Masaaki Minami1*, Syun Torii1, Michio Ohta2

1Department of Bacteriology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan
2School of Nursing, Sugiyama Jyogakuen University, Nagoya, Japan
Email: *minami@med.nagoya-cu.ac.jp

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Abstract

In Streptococcus pyogenes, we have described the two-component signal transduction sensor and regulatory systems, CovR/S affect the antimicrobial susceptibility including penicillin G before. But the mechanism how two-component sensor protein CovS modulates penicillin G susceptibility has not been elucidated till date. This study aimed to determine how the CovS affected penicillin G susceptibility in Streptococcus pyogenes by northern blot analysis. At first, we investigated the covS mRNA expression under penicillin G induction. We found that the decrease of covS mRNA expression under Penicillin G stimulation. Next we investigated the expression of cell wall synthesis gene, pbp2a and glmM with use of covS knockout mutants from emm1 Streptococcus pyogenes strain 1529. We found that the cell-wall synthesis gene expression of the ΔcovS mutant strain were lower than that of the wild-type strain. Furthermore the expression of glmM mRNA gene was lower than the expression of pbp2a mRNA gene in the ΔcovS mutant strain. The covS-complemented strain almost restored the mRNA expression compared to ΔcovS mutant strain. The two-component sensor protein CovS affects the susceptibility to penicillin G in Streptococcus pyogenes by modulation of cell-wall synthesis.

Keywords

Streptococcus Pyogenes, CovS, pbp2a, glmM

1. Introduction

Streptococcus pyogenes is a gram-positive bacterium that infects the upper respiratory tract, including the tonsils and pharynx, and is responsible for post-infectious diseases, such as rheumatic fever, glomerulonephritis, and streptococcal toxic shock-like syndrome [1]. Although the pathogenesis of Streptococcus pyogenes is unclear, many virulent proteins are considered to be causative factors.

In the basic model of two-component systems, interaction of an appropriate extracellular stimulus with the sensor histidine kinase alters the phosphorylation state of its cytoplasmic domain [2]. The prototypic sensor protein has kinase and/or phosphatase activity for a cognate regulator protein; phosphorylation (or dephosphorylation) of the regulator controls its activity as a transcriptional activator or repressor for one or more target genes [3]. In many cases, signaling through a single two-component system results in a coordinated change in expression of multiple genes whose products play a role in adaptation to a particular environment [2]. Two-component systems CovR/S in *Streptococcus pyogenes* has been shown to regulate expression of several virulence determinants, including the hyaluronic acid capsule, streptolysin S, and streptokinase [2] [3]. Microarray transcriptional profiling studies suggest that CovR/S regulates expression, directly or indirectly, of 15% of *Streptococcus pyogenes* genes [2] [3].

We described that CovR/S system may play a role in antibiotics susceptibility before [4]. However, the precise mechanism between CovS and penicillin G susceptibility has not yet been elucidated. Here, we focused on the role of cell-wall synthesis and evaluated whether CovS modulates cell-wall synthesis in *Streptococcus pyogenes*.

2. Materials and Methods

2.1. Bacterial Strains and Culture Condition

M1 serotype (*emm*1 genotype) *Streptococcus pyogenes* strains 1529 used in this study were clinical isolates from hospital patients in Japan with invasive *Streptococcus pyogenes* infections [4]. We also used *covS* (∆*covS*) knockout and *covS*-complemented (∆*covS* comp) mutants from *Streptococcus pyogenes* strains 1529 [4]. *Streptococcus pyogenes* was usually cultured in 5 mL of Brain-Heart Infusion Broth (Eiken Chemical Co., Tokyo, Japan) containing 0.3% yeast extract (Difco Laboratories, Detroit, MI, USA) (BHI-YE) for 18 - 24 h at 37°C without agitation. The following antibiotic concentrations were used when appropriate: penicillin G (Sigma Chemical Co, St. Louis, MO, USA), 0.1 μg/mL, spectinomycin (Sigma Chemical Co), 100 μg/mL.

2.2. RNA Isolation and Northern Blot Analysis

Total RNA was extracted and purified as described previously [5] [6]. In brief, bacterial cells were cultured in 5 mL of BHI-YE. The cells were harvested at approximately 0.6 absorbance (log phase) at O.D.660. When induction study, the cells were cultured with penicillin G for further 15 minutes. Total RNA was extracted and purified with ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). Approximately 2 μg of each of the total RNA preparation was electrophoresed onto a 1% agarose gel containing 1.1 M formaldehyde (Wako Pure Chemical Industries). RNAs were transferred onto a Hybond -N+ membrane (GE Healthcare, Waukesha, WI, USA). The DNA probes (*covS* (Spy0337)), *pbp2a* (Spy2059), *glmM* (Spy1038), and *rpsL* (Spy0271)) were amplified with oligonucleotide primers as follows: *csrS*-n4 (5’-ACAAGGGCTATTGACCCAC-3’) and *csrS*-c1 (5’-GCACCTGCACTAATCCTTTGACTGGG-3’); *pbp2a*-f1 (5’-AGCACTCAGGCTATTACAT-3’) and *pbp2a*-r1 (5’-TCCAAATAGATTGGGCTTTA-3’); *glm*-f1 (5’-CAGCACGTGATCACCGTCAACA-3’) and *glm*-r1 (5’-TATACGATCACCCTACAACA-3’); *rpsL*-f1(5’-GAATGTAGATGCTACCAATTAACA-3’) and *rpsL*-r1 (5’-TTTACGACTATTCTCTTCTTACCCA-3’) [5]. These probes were 32P-labeled using the random primer DNA labeling kit version 2 (Takara Bio, Ohtsu, Japan). The membranes were then autoradiographed and analyzed at room temperature with a bioimaging analyzer (BAS-1800II; Fujifilm, Tokyo, Japan). The express of *rpsL* mRNA was evaluated as internal control. The data from the bioimaging analyzer were calculated quantitatively.

2.3. Statistical Analysis

Statistical significance between the mean values was determined by one-way analysis of variance. A confidence interval with a *p* value of <0.05 was considered to be significant. The compared experiments were repeated a minimum of three times to improve the resulting data.

3. Result

3.1. Northern Blot Analysis Revealed That the Expression Level of *covS* mRNA Was Decreasing under PCG Induction in 1529 Wild-Type Strain

In our previous study, we suggested that CovS contributed to the effect of penicillin G treatment [4]. Hence, us-
ing northern blot analysis, we first analyzed whether penicillin G induced the expression of \textit{covS}. As expected, after penicillin G treatment, the level of \textit{covS} mRNA was increasing in comparison with the level without penicillin G induction (Figure 1).

3.2. Northern Blot Analysis Revealed That the Expression Level of \textit{pbp2a} mRNA Was Decreasing in the 1529\textit{covS} Mutant Strain

Next we evaluated the expression of \textit{pbp2a} mRNAs among \textit{Streptococcus pyogenes} 1529 \textit{ΔcovS} mutant strains. Thus, we have confirmed the change of \textit{pbp2a} mRNA expression in both wild-type, 1529 \textit{ΔcovS} strain, and \textit{covS}-complemented strains by northern blot analysis. Figure 2 shows that the expression of \textit{pbp2a} mRNA in the 1529 \textit{ΔcovS} mutant strain was lower than that in the wild-type strain.

3.3. Northern Blot Analysis Revealed That the Expression Level of \textit{glmM} mRNA Was Decreasing in the 1529\textit{covS} Mutant Strain

Furthermore, we evaluated the expression of \textit{glmM} mRNAs among \textit{Streptococcus pyogenes} 1529 \textit{ΔcovS} mutant strains. Thus, we have confirmed the change of \textit{glmM} mRNA expression in both wild-type, 1529 \textit{ΔcovS} strain, and \textit{covS}-complemented strains by northern blot analysis. Figure 3 shows that the expression of \textit{glmM} mRNA in the 1529 \textit{ΔcovS} mutant strain was lower than that in the wild-type strain.

![Figure 1](image1.png)  
**Figure 1.** The expression of \textit{covS} and \textit{rpsL} mRNA under penicillin G induction by northern blot analysis. PCG: penicillin G. Asterisk indicates \( p < 0.05 \).

![Figure 2](image2.png)  
**Figure 2.** The expression of \textit{pbp2a} and \textit{rpsL} mRNA in 1529\textit{covS} derived strains by northern blot analysis. Wild: 1529 wild-type, \textit{ΔcovS}: 1529 \textit{covS} knockout mutant, \textit{ΔcovS} comp: 1529 \textit{covS}-complemented mutant. Asterisks indicate \( p < 0.05 \).
4. Discussion

In this study, we clarified that the mechanism between the *Streptococcus pyogenes* two component sensor protein CovS and penicillin G susceptibility. We have demonstrated that *covS*-inactivation in *Streptococcus pyogenes* was associated with increased penicillin G susceptibility before. Although this mechanism has been unclear, we have suggested two hypotheses from previous investigations [4]. One hypothesis is that CovS may affect the bacterial growth [4]. Previous report showed that a Δ*covS* mutant strain had lower growth ability than wild-type strain [7]. Antibiotic stress may result in the wideness of the growth differences between wild-type and *covS* mutant strains. Another hypothesis is that CovS may affect the function of PBP2a [4]. The *php2a* gene encodes transpeptidase which plays a role in cross linking of cell-wall [8]. Previous report represented that Δ*covR* mutant increased the expression of *php2a* mRNA compared to wild-type strain in microarray study [2]. CovS regulates the expression of CovR negatively [7]. The lack of *covR* may decrease the PBP2a activity via the uptake of CovR expression and may result in the weakness of cell-wall structure [4]. We clarified this point in this study. Penicillin G decreased the expression of *covS* gene in 1529 wild-type strain. The Δ*covS* mutant strain had lower cell-wall synthesis gene *php2a* gene and *glmM* gene than the wild-type strain. Many factors influence antimicrobial susceptibility, and each factor is subjected to complex cross-talk regulation. Our results revealed part of the penicillin G susceptibility in *Streptococcus pyogenes*.

Penicillin-binding proteins (PBPs) are membrane-bound D, D-peptidases that have evolved from serine proteases [9]. These enzymes catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall [8]. β-lactam antibiotics, which are substrate analogues, covalently bind to the PBP active site serine and inactivate PBPs at concentrations that are about the same as the MICs [9]. *Streptococcus pyogenes* possesses five major PBPs [8]. The high-molecular-weight PBPs (mass > 60 kDa) are the critical antibiotic targets [9]. PBPs 1, 2, and 3, which have high affinity for most β-lactam antibiotics, are essential for cell growth and survival of susceptible strains [8]. Binding of β-lactams by these PBPs is lethal [8]. Previous study revealed that most of the radioactive penicillin was bound to PBP 2a/b both in *vivo* and *in vitro* and that the source of the amounts of radioactivity transferred to PBP3 was PBP 2 [8]. Although low-molecular-weight PBP 4 and 5 may be important in normal cell wall synthesis and participate to a limited extent resistance, they are not considered a critical target and are dispensable [8]. Thus we focused on *php2a* gene expression in this investigation.

UDP-N-acetylgalactosamine (UDP-GalNAc) is an essential common precursor for synthesis of bacterial cell-wall peptidoglycan and outer membrane lipopolysaccharide [10] [11]. It is a directly glycosyl donor of linker unit and involved in the attachment of galactofuran and peptidoglycan [10] [11]. In bacteria, UDP-GlcNAc is synthesized from the glycolytic intermediate D-fructose-6-phosphate by four reactions catalyzed by three enzymes: glucosamine-6-phosphate synthase (GlmS), phosphogluco amine phosphogluco amine mutase (GlmM; EC 5.4.2.10) and the bifunctional enzyme glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-
phosphate uridyltransferase (GlmU) \([10] \[11]\). Especially, GlmM catalyzes the inter conversion of glucosamine-6-phosphate to glucosamine-1-phosphate, an essential step in the biosynthetic pathway leading to the formation of the peptidoglycan precursor UDP-GlcNAc \([10] \[11]\). A mutation in the \(glmM\) gene affects both peptidoglycan and lipopolysaccharide synthesis in \(Escherichia coli\) \([10] \[11]\). Homologs of \(glmM\) in \(Staphylococcus aureus\) and \(Streptococcus gordonii\) are associated with sensitivity to antibiotics \([10] \[11]\). Thus we supposed that the expression of \(glmM\) gene in \(Streptococcus pyogenes\) were also associated with penicillin G susceptibility in this study.

One mechanism for adaptation to changing environments is through two-component systems, a family of proteins that are widely distributed among many bacterial genera \([2] \[3]\). Two-component systems allow sensing of specific environmental signals through a sensor histidine kinase that is usually associated with the cell membrane \([2] \[3]\). Various antibiotic stresses have been reported to be recognized by other bacterial two-component systems. VraSR from \(Staphylococcus aureus\) is induced by bacitracin and vancomycin and also by other cell-wall antibiotics such as D-cycloserine \([12]\). A VraSR knockout strain shows a significant increase in sensitivity to the antibiotics it senses \([12]\). Although we demonstrated that two-component system CovR/S affected antibiotic susceptibility and that not only PBP2a but also GlmM play a role in penicillin G susceptibility via CovR/S system, the comparative investigation of other two-component systems in \(Streptococcus pyogenes\) may be also necessary according to the antibiotic susceptibility pattern.

5. Conclusion

In summary, we clarified that the mechanism between the \(Streptococcus pyogenes\) two-component sensor protein CovS and penicillin G susceptibility by modulation of cell-wall synthesis. In particular, CovS may play an important role in the enzymatic activity of not only membrane-bound transpeptidase but also phosphoglucominomutase. Further investigations are needed to elucidate the mechanism of antimicrobial susceptibility in \(Streptococcus pyogenes\) via two-component signal transduction sensor and regular system pathways.

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