Phosphodiesterase Activity of CvfA Is Required for Virulence in \textit{Staphylococcus aureus}*

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We previously identified the \textit{cvfA} gene (SA1129) as a novel virulence regulator in \textit{Staphylococcus aureus} using the silkworm infection model. The \textit{cvfA} gene, which is conserved among various pathogenic bacteria, contributes to the expression of the \textit{agr} locus, a global virulence regulator that controls the expression of genes encoding various exoenzymes, such as hemolysin. Cvfa protein has a transmembrane domain, an RNA binding domain (KH domain), and a metal-dependent phosphohydrolase domain (HD domain). We report here the purification of recombinant Cvfa protein from a membrane fraction of \textit{Escherichia coli} by measuring its phosphodiesterase activity. Purified Cvfa protein hydrolyzed the phosphodiester linkage of 2',3'-cylic AMP, 2',3'-cylic GMP, and 2',3'-cylic phosphate at the 3'-terminal of RNA in the presence of Mn$^{2+}$. Cvfa mutant proteins with amino acid substitutions in the HD domain had significantly decreased phosphodiesterase activity. Furthermore, mutated \textit{cvfA} genes encoding proteins with low phosphodiesterase activity did not complement the decreased hemolysin production or the attenuated killing ability against silkworms in the \textit{cvfA} deletion mutant. These results suggest that the phosphodiesterase activity of Cvfa protein is required for virulence in \textit{S. aureus}.

\textit{Staphylococcus aureus} is an opportunistic pathogen in humans that causes various diseases, such as localized skin infection, septic arthritis, osteomyelitis, endocarditis, and toxic shock syndrome (1). These diseases are caused by the combination of various virulence factors expressed by \textit{S. aureus}, which contain cell surface proteins to adhere to host tissues and exotoxins such as hemolysin, protease, and nuclease to destroy host tissues (2–4). Several genes regulate the expression of genes encoding virulence factors in \textit{S. aureus}, such as the \textit{agr} locus (5, 6), \textit{sara} (7), \textit{saeRS} (8), \textit{srrAB} (9), and \textit{arlRS} (10). These genes are assumed to interact with each other and to regulate the expression of virulence genes. The overall picture of the regulatory cascades, however, is obscure (11).

Based on our assumption that there are novel virulence regulatory genes among the uncharacterized genes of \textit{S. aureus}, we screened gene-disrupted mutants using a previously reported silkworm-infection model (12). Using this strategy, we identified novel virulence genes named \textit{cvfA}, \textit{cvfB}, and \textit{cvfC}, whose mutation resulted in an attenuated killing ability against silkworms (13, 14). The \textit{cvfA}, \textit{cvfB}, and \textit{cvfC} genes are conserved among various pathogenic bacteria, and also contribute to virulence in mice. In \textit{S. aureus}, the \textit{cvfA} gene is required for transcriptional expression of the \textit{agr} locus, and for the production of exotoxins such as hemolysin and protease (13). We also identified the \textit{sarZ} gene, a novel virulence regulator, as a multicopy suppressor of decreased hemolysin production in the \textit{cvfA} deletion mutant (15). The \textit{cvfA} gene is considered to promote the expression of the \textit{sarZ} gene, followed by activation of the expression of the \textit{agr} locus, and to contribute to hemolysin production (15). Moreover, under the genetic background of the \textit{agr}-null mutation, \textit{cvfA} disruption attenuates virulence in silkworms (13), suggesting that the \textit{cvfA} gene contributes to virulence via both \textit{agr}-dependent and \textit{agr}-independent pathways in \textit{S. aureus}. Thus, Cvfa is thought to function upstream of the various virulence regulatory systems. The biochemical features of Cvfa protein, however, remained to be determined.

In the present study, we purified Cvfa protein and determined that it has phosphodiesterase activity that hydrolyzes 2',3'-cylic AMP and 2',3'-cylic GMP into 3'-AMP and 3'-GMP, respectively. Furthermore, analysis of mutated Cvfa proteins with low phosphodiesterase activity suggests that phosphodiesterase activity of Cvfa is required for virulence in \textit{S. aureus}.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The JM109 strain of \textit{Escherichia coli} was used as a host for pET-11a, pSF151, pND50, pMtinT3, and their derivatives. \textit{E. coli} strains transformed with the plasmids were cultured at 37 °C in Luria-Bertani broth containing 50 μg/ml ampicillin or 50 μg/ml kanamycin or 12.5 μg/ml chloramphenicol. \textit{S. aureus} strain RN4220, NCTC8325-4, and their derivative strains were aerobically cultured in tryptic soy broth at 37 °C; when needed, 12.5 μg/ml chloramphenicol was added. The details of the bacterial strains and plasmids used in this study are shown in Table 1.

**DNA Manipulation and Deletion Mutagenesis**—Transformation of \textit{E. coli}, extraction of plasmid DNA from \textit{E. coli}, and polymerase chain reaction were performed as previously reported (16). The primers used in this study are listed in Table 2. \textit{S. aureus} was transformed with plasmid DNA using electroporation (17). To construct plasmids for the overproduction of
CvfA protein, PCR amplification was performed using primers FCvfA and RCvfA with RN4220 genomic DNA as a template. The amplified DNA fragment was inserted into the NdeI and BamHI sites of pET-11a, resulting in pCvfA.

Site-directed mutagenesis was performed according to the method of Li and Wilkinson (18). The cvfA gene, amplified by PCR using primers FccvfA and RccvfA with RN4220 genomic DNA as a template, was cloned into the EcoRI and BamHI sites of pUC119. Using primer pairs for mutagenesis (e.g. FH338A and RH338A) and pUC119-cvfA as the template, mutant strands were synthesized by thermal cycling, and then template plasmid was digested with DpnI. The E. coli JM109 strain was transformed with the synthesized mutated plasmids (pUC119-mutant-cvfA). The plasmids were extracted and sequenced to confirm the desired cvfA point mutation. Using primer pairs FCvfA and RCvfA and the mutated plasmid as the template, PCR amplification was performed. The amplified DNA fragments were cloned into the NdeI and BamHI sites of pET-11a, resulting in pH338A, pH367A, pD368A, pK371A, and pD423A.

### TABLE 1

A list of bacterial strains and plasmids used in this study

| Strain or plasmid | Genotypes or characteristics | Source or Ref. |
|-------------------|------------------------------|----------------|
| Strains
| E. coli
| JM109 | General purpose host strain for cloning |
| BL21(DE3)pLysS | General purpose host strain for expression of recombinant proteins |
| S. aureus | NCTC8325-4, restriction mutant |
| RN4220 | NCTC8325 cured of φ11, φ12, and φ13 |
| MP1129 | MP1129 RN4220 cvfA::phleo |
| NCTC8325 | NCTC8325 cured of φ11, φ12, and φ13 |
| NCTC8325-4 | NCTC8325-4 |
| CKP1129 | This study |
| Plasmids
| pET-11a | T7 promoter based expression vector, Amp' |
| pCvfA | pET-11a with cvfA (wt) |
| pH338A | pET-11a with mutated cvfA (H338A) |
| pH367A | pET-11a with mutated cvfA (D368A) |
| pD368A | pET-11a with mutated cvfA (D368A) |
| pK371A | pET-11a with mutated cvfA (K371A) |
| pD423A | pET-11a with mutated cvfA (D423A) |
| pSF151 | S. aureus integration vector; Km' |
| pSF1129-phleo | pSF1129 with cvfA's 3' arm |
| pMutinT3 | E. coli IPTG inducible expression vector; Amp', Erm' |
| pND50 | E. coli - S. aureus shuttle vector; Cm' |
| pMN | S. aureus IPTG inducible expression vector; Cm' |
| pPsx:cvfA | pMN with cvfA (wt) |
| pPsx-H338A | pMN with mutated cvfA (H338A) |
| pPsx-H367A | pMN with mutated cvfA (D368A) |
| pPsx-D368A | pMN with mutated cvfA (D368A) |
| pPsx-K371A | pMN with mutated cvfA (K371A) |
| pPsx-D423A | pMN with mutated cvfA (D423A) |
| pSF151 | This study |
| pSF1129-phleo | This study |
| pMutinT3 | This study |
| pND50 | This study |
| pMN | This study |
| pPsx-H338A | This study |
| pPsx-H367A | This study |
| pPsx-D368A | This study |
| pPsx-K371A | This study |
| pPsx-D423A | This study |
| pSF151 | This study |
| pSF1129-phleo | This study |
| pMutinT3 | This study |
| pND50 | This study |
| pMN | This study |
| pPsx-H338A | This study |
| pPsx-H367A | This study |
| pPsx-D368A | This study |
| pPsx-K371A | This study |
| pPsx-D423A | This study |

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### TABLE 2

PCR primers used in this study

| Target | Primer | Sequence (5'-3') |
|--------|--------|-----------------|
| cvfA   | FccvfA | CGAATTATGAAATTTATGGTCCTG |
|        | RccvfA | GGTATGACAAATAAAATACCAAAATTCG |
|        | FC1129 | GCCGCCAAATTCTCACTAAGGATTGTTGTT |
|        | RC1129 | GCCGCCAAAACATTCGGGTTGTT |
|        | pET-11a | T7 promoter based expression vector, Amp' |
|        | pCvfA | pET-11a with cvfA (wt) |
|        | pH338A | pET-11a with mutated cvfA (H338A) |
|        | pH367A | pET-11a with mutated cvfA (D368A) |
|        | pD368A | pET-11a with mutated cvfA (D368A) |
|        | pK371A | pET-11a with mutated cvfA (K371A) |
|        | pD423A | pET-11a with mutated cvfA (D423A) |
|        | pSF151 | S. aureus integration vector; Km' |
|        | pSF1129-phleo | pSF151 with cvfA's 3' arm |
|        | pMutinT3 | E. coli IPTG inducible expression vector; Amp', Erm' |
|        | pND50 | E. coli - S. aureus shuttle vector; Cm' |
|        | pMN | S. aureus IPTG inducible expression vector; Cm' |
|        | pPsx:cvfA | pMN with cvfA (wt) |
|        | pPsx-H338A | pMN with mutated cvfA (H338A) |
|        | pPsx-H367A | pMN with mutated cvfA (D368A) |
|        | pPsx-D368A | pMN with mutated cvfA (D368A) |
|        | pPsx-K371A | pMN with mutated cvfA (K371A) |
|        | pPsx-D423A | pMN with mutated cvfA (D423A) |
|        | pSF151 | This study |
|        | pSF1129-phleo | This study |
|        | pMutinT3 | This study |
|        | pND50 | This study |
|        | pMN | This study |
|        | pPsx-H338A | This study |
|        | pPsx-H367A | This study |
|        | pPsx-D368A | This study |
|        | pPsx-K371A | This study |
|        | pPsx-D423A | This study |

CvfA Phosphodiesterase Activity in Staphylococcal Virulence
CvfA Phosphodiesterase Activity in Staphylococcal Virulence

To regulate the expression of CvfA proteins under the control of isopropyl 1-thio-β-β-galactopyranoside (IPTG) within *S. aureus* cells, the plasmid pMN was constructed by fusing the pMutinT3 and pND50 plasmids. The pND50 fragment lacking the multicloning site was amplified by PCR using primers FpND50 and RpND50. The pMutinT3 fragment containing the terminators *t*<sub>A</sub> and *t*<sub>B</sub>, Pspac promoter, *lacZ*, and *lacI* gene-coding region was amplified by PCR using primers FpMUTIN and RpMUTIN. These two DNA fragments were digested by KpnI and SpeI, then ligated with *E. coli* ligase, resulting in the pMN plasmid.

To construct plasmids containing wild-type and mutant cvfA genes for the complementation experiments, PCR amplification was performed using primers FC1129 and RC1129 with pUC119-cvfA or pUC119-mutant-cvfA as the templates. The amplified DNA fragment was inserted into the EcoRI and BamHI sites of pMN, resulting in pPspac-CvfA and -H338A, -H367A, -D368A, -K371A, and -D423A.

**Purification of Recombinant CvfA Proteins**—*E. coli* BL21(DE3)pLysS was transformed with plasmid pCvfA and grown in Luria-Bertani broth containing 50 μg/ml ampicillin at 37 °C to an *A*<sub>600</sub> = 0.5. Recombinant CvfA proteins were induced with 1 mM IPTG for 2 h. Bacterial cells were harvested by centrifugation, resuspended in lysis buffer (50 mM HEPES-KOH, pH 8.0, 250 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol), and lysed by sonication. The resulting homogenate was centrifuged at 6,000 × *g* for 10 min, the resulting supernatant was centrifuged at 100,000 × *g* for 30 min, and the pellet was washed once with lysis buffer. The resulting pellet was obtained as the membrane fraction (Fraction I). The membrane fraction was resuspended in buffer C′ (50 mM HEPES-KOH, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) containing 1.5% n-octyl glucoside and 0.5 M KCl, and sonicated three times for 15 s (OPC1) using a Branson Sonifier 450, and then centrifuged at 100,000 × *g* for 30 min and a pellet was obtained (Fraction II). The pellet was resuspended in buffer C′ containing 2.5% Tween 20 and sonicated four times for 30 s (OPC2) and centrifuged at 100,000 × *g* for 30 min, the resulting supernatant was obtained as the solubilized fraction (Fraction III). Fraction III was loaded onto a DEAE-toyopearl 650 (TOSOH, Tokyo, Japan) column (1.5 × 3 cm) preequilibrated with buffer C′ containing 0.5% CHAPS, and washed with buffer C′ containing 0.5% CHAPS and 0.5 M KCl. Phosphodiesterase activity was not detected in the wash fraction. The activity was then eluted with buffer C′ containing 2.5% Tween 20 and 0.5 M KCl (Fraction IV). Fraction IV was dialyzed against the buffer C′ containing 2.5% Tween 20 and loaded onto a MonoQ HR5/5 column (Amersham Biosciences) and eluted with a linear gradient from 0 to 0.8 M KCl. Each fraction was analyzed by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. Fractions I to III containing the various mutated CvFA proteins were prepared in a similar way. The protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

**Enzymatic Assays**—Phosphodiesterase activity was measured in a reaction mixture containing 50 mM HEPES-KOH (pH 9.0), 0.6 mM KCl, 1 mM MnCl<sub>2</sub>, and 16 mM bis-p-nitrophenyl phosphate (bis-pNPP). After 20 min incubation at 37 °C, the reaction was stopped by adding 10 mM EDTA and the amount of *p*-nitrophenol produced was determined from the absorbance at 405 nm. Phosphatase activity was measured in a similar way using pNPP as a substrate.

Phosphodiesterase activity with natural substrates (various 2′,3′- and 3′,5′-cyclic nucleoside monophosphates from *Sigma*) was measured as described previously (19). CvFA protein and 20 mM cyclic nucleotide were incubated for 60 min at 37 °C in a reaction mixture containing 50 mM HEPES-KOH (pH 9.0), 0.6 mM KCl, 1 mM MnCl<sub>2</sub>, and the reaction was stopped by adding 10 mM EDTA. The products were analyzed by thin layer chromatography (TLC) on cellulose F plates (Merck, Darmstadt, Germany) in solvent A (saturated ammonium sulfate, 3 M sodium acetate, isopropyl alcohol; 80:6:2). The reaction products and the nucleotide standards were visualized under ultraviolet light, and the signal intensity was quantified using Image Gauge software (FUJI PHOTO FILM CO., LTD., Tokyo, Japan).

Staphylococcal RNA containing guanosine 2′,3′-cyclic phosphate in its 3′-terminal nucleuside ([Np]<sub>G</sub>) was prepared as previously described with slight modification (20). Briefly, total RNA prepared from *S. aureus* RN4220 was digested with 145 units of RNase T1 and the 3′-terminal phosphates were removed by treatment with alkaline phosphatase (TaKaRa Bio, Shiga, Japan), followed by phenol extraction and ethanol precipitation. Resulting oligonucleotides was incubated with [γ<sup>32</sup>P]pCp (GE Healthcare) and T4 RNA ligase (TaKaRa Bio) to produce (Np)<sub>G</sub>−p<sup>−</sup>(Np)<sub>G</sub>−pCp. To generate (Np)<sub>G</sub>p<sup>−</sup>−p<sup>−</sup>(Np)<sub>G</sub>pCp was digested with 1 unit of RNase T1, followed by treatment with alkaline phosphatase to dephosphorylate (Np)<sub>G</sub>−p<sup>−</sup>p<sup>−</sup> generated by RNase T1. The sample was extracted with phenol, and the aqueous phase was applied to a CENTRI-SEP column (Princeton Separations, Inc., NJ) equilibrated with 20 mM ammonium acetate (pH 5.5). The average length of the resulting (Np)<sub>G</sub>p<sup>−</sup>−p<sup>−</sup>p<sup>−</sup> was 50 bases from urea-polyacrylamide gel electrophoresis analysis. CvFA protein and 200 nM (Np)<sub>G</sub>p<sup>−</sup>−p<sup>−</sup>p<sup>−</sup> were incubated for 60 min at 37 °C in a reaction mixture containing 50 mM HEPES-KOH (pH 9.0), 0.6 M KCl, 1 mM MnCl<sub>2</sub>, followed by phenol extraction and ethanol precipitation. The resulting products were digested with nuclelease P1 (Roche, Germany), separated by TLC as described above, and analyzed by autoradiography. The marker pG−p was prepared by phosphorylating G−p (2′,3′-cyclic GMP) using T4 polynucleotide kinase (TaKaRa Bio), and its position on the TLC plate was detected under ultraviolet light.

**Construction of the cvfa-deletion Mutant**—To construct a targeting vector for disruption of the cvfa gene, the cvfa 5−arm or 3−arm DNA fragment, the region located just upstream or downstream of the cvfa gene, respectively, was amplified by PCR with RN4220 genomic DNA as a template, using primers P1 and P2 or P3 and P4, respectively. The marker, phleomycin resistance gene, was amplified by PCR using primers FpHleo and RpHleo with a ump cassette as a template (21). The three
amplification DNA fragments were inserted into the XbaI and EcoRI sites of pSF151 in the following order: 5'-arm, pleomycin resistance gene, and 3'-arm, resulting in pSF1129-phleo. A strain resistant to both kanamycin and pleomycin was obtained by transforming the RN4220 strain with pSF1129-phleo. Among these strains, we screened a strain that becomes sensitive to kanamycin but resistant to pleomycin, resulting in the MP1129 strain, in which the cvfA gene was replaced by the pleomycin resistance gene. Disruption of the cvfA gene was confirmed by Southern blot analysis using the PCR-amplified fragment, ranging from the cvfA coding region to the 3'-arm, as a probe. To construct the cvfA-deletion mutant in the NCTC8325-4 strain, namely CKP1129, phage transduction was performed as described previously (22). Phage 80α lysates of the MP1129 strain were used to infect NCTC8325-4. Similarly, plasmids were transferred to recipient strains by phage transduction.

Hemolytic Activity Assay—Hemolytic activity was measured using the previously described method (23). Briefly, a supernatant of the culture at 18 h after inoculation was incubated with sheep red blood cells at 37 °C for 1 h. The reaction mixture was centrifuged (1000 × g, 5 min) and the increase in the A450 of the supernatant was determined. The hemolytic activity was expressed as hemolytic units corresponding to the reciprocal of the dilution of supernatant that yielded 50% lysis of the erythrocytes.

Silkworm Infection Experiment—The silkworm infection experiment was performed according to the previously established method (13). Silkworms were raised from fertilized eggs to fifth-instar larvae in our laboratory. The hatched fifth-instar larvae were fed antibiotic-free artificial food (Silkworm food, Katakura Industries, Japan) for 1 day. Bacterial suspensions (0.05 ml, 4 × 10^7 colony forming units) were injected into the hemolymph of the larvae through the dorsal surface using a 27-gauge needle (Terumo, Tokyo, Japan). Overnight cultures in the tryptic soy broth containing 0.5 mM IPTG were used for the experiments. The injected larvae were maintained without food in a safety cabinet (BHC-1303 II A; Airtech Japan, Tokyo, Japan) at 27 °C with 50% humidity. The larvae were injected 10 mM IPTG (0.05 ml) every 24 h and survival was monitored.

RESULTS

Purification of CvfA Protein from E. coli Membrane—CvfA protein has a transmembrane domain, an RNA binding domain (KH domain), and metal-dependent phosphohydrolase domain (HD domain) (Fig. 1A). The amino acid sequence of the CvfA HD domain is conserved among various pathogenic bacteria. In particular, there is a greater than 99% sequence identity of the consensus residues that are considered to be important for the phosphohydrolase activity (Fig. 1B) (24–26). Therefore, we hypothesized that CvfA protein has phosphohydrolase activity, and attempted to purify the recombinant CvfA protein by measuring this activity.

First, we lysed E. coli expressing S. aureus CvfA protein by sonication, and prepared the membrane fraction. In the membrane fraction prepared from E. coli expressing CvfA, the hydrolysis activity against bis-pNPP, an artificial phosphodies-
gave a single band with a molecular mass of 59 kDa, which is the estimated size of the CvfA protein, on SDS-PAGE (Fig. 2B). The final step of MonoQ chromatography revealed that the phosphodiesterase activity co-migrated with the CvfA protein (Fig. 2C and D). Based on these results, we concluded that CvfA protein was purified as a phosphodiesterase.

**Biochemical Characterization of Phosphodiesterase Activity of CvfA Protein**—Some enzymes possessing the HD domain not only has phosphodiesterase activity but also phosphatase activity (19, 24). We next examined whether CvfA had phosphatase activity.

**TABLE 3**

| Fraction | Protein | Total activity | Yield | Specific activity |
|----------|---------|----------------|-------|------------------|
| I Membrane | 25 | 3.25 | 100 | 1.4 |
| II 1.5% OG-washed membrane | 7.8 | 23.3 | 67 | 3.0 |
| III 2.5% Tween 20 solubilized | 2.3 | 15.5 | 44 | 6.8 |
| IV DEAE-toyopearl column | 1.08 | 18.4 | 53 | 17.1 |
| V MonoQ column | 0.37 | 8.39 | 24 | 22.9 |

OG, n-octyl-glucoside.

**FIGURE 2.** Purification of recombinant CvfA protein from *E. coli* membrane. A, phosphodiesterase activity in the membrane fraction (Fraction I) and in the membrane washed by n-octyl glucoside (Fraction II) prepared from *E. coli* harboring the CvfA expression vector or empty vector. Bis-pNPP was used as a substrate for phosphodiesterase. B, SDS-PAGE analysis of proteins at each step of purification. The gel was stained with Coomassie Brilliant Blue. Arrowhead indicates CvfA protein (59 kDa). C, an elution profile of MonoQ chromatography. Filled circles indicate phosphodiesterase activity with bis-pNPP as a substrate. Open triangles indicate protein concentration. The dotted line shows the KCl concentration gradient. D, SDS-PAGE of MonoQ fractions (26–35). The gel was stained with Coomassie Brilliant Blue.

**FIGURE 3.** Characterization of CvfA phosphodiesterase activity with bis-pNPP as a substrate. A, CvfA protein was incubated with a phosphodiesterase substrate (bis-pNPP, 16 mM) or a phosphatase substrate (pNPP, 16 mM) in buffer containing 50 mM HEPES-KOH (pH 9.0) and 1 mM MnCl₂, and the amount of p-nitrophenol produced was measured. B, dependence on bis-pNPP concentration of CvfA phosphodiesterase activity was analyzed in buffer containing 50 mM HEPES-KOH (pH 9.0) and 1 mM MnCl₂. C, metal dependence of CvfA phosphodiesterase activity was analyzed at pH 9.0. Reaction mixtures contained 16 mM bis-pNPP and 1 mM MnCl₂, NiCl₂, CoCl₂, CuCl₂, ZnCl₂, CaCl₂, or MgCl₂.
activity. When CvfA was incubated with a phosphodiesterase substrate (bis-pNPP), p-nitrophenol was produced in a dose-dependent manner. On the other hand, when CvfA was incubated with a phosphatase substrate (pNP), p-nitrophenol was not produced (Fig. 3A). This result suggests that CvfA does not have phosphatase activity. Scatchard plot analysis demonstrated that the $K_m$ value of the hydrolysis activity of CvfA against bis-pNPP was 30 mM and the $k_{cat}$ was 0.7/s (Fig. 3B, Table 4). CvfA phosphodiesterase activity requires Mn$^{2+}$ or Ni$^{2+}$ (Fig. 3C), and the optimal pH was 9.0 in buffer containing 1 mM Mn$^{2+}$ (data not shown).

**Phosphodiesterase Activity with Natural Substrates**—To determine the natural compounds hydrolyzed by CvfA, we examined whether CvfA has phosphohydrolase activity against cyclic nucleotides, which are known substrates of phosphodiesterase that contain the HD domain (19). CvfA was incubated with various cyclic nucleotides and the reaction products were analyzed by TLC. When CvfA was incubated with 2',3'-cAMP, 3'-AMP was produced (Fig. 4A). Similarly, incubation with 2',3'-cGMP produced 3'-GMP (Fig. 4B). On the other hand, when CvfA was incubated with 3',5'-cAMP, 3',5'-cGMP, or 2',3'-cCMP, the hydrolyzed products were not detected (Fig. 4, A–C). These results suggest that CvfA has phosphodiesterase activity against 2',3'-cAMP and 2',3'-cCMP; but not 3',5'-cAMP, 3',5'-cGMP, and 2',3'-cGMP. As with bis-pNPP, CvfA phosphodiesterase activity against 2',3'-cAMP requires Mn$^{2+}$ or Ni$^{2+}$ (data not shown). Scatchard plot analysis demonstrated that the $K_m$ value of the hydrolysis activity of CvfA against 2',3'-cAMP and 2',3'-cGMP was 17 and 15 mM, respectively (Fig. 4, D and E, Table 4).

In bacteria, di-cGMP is used as a signaling molecule to regulate various cellular functions including virulence (27). Di-cGMP is hydrolyzed by enzymes possessing the HD domain (26), but CvfA did not hydrolyze di-cGMP (data not shown).

**Phosphodiesterase Activity against 2',3'-Cyclic Nucleotide Structure at the 3'-Terminal of RNA**—The 2',3'-cyclic nucleotide is produced at the 3'-terminal of RNA when cleaved by endonucleases. Because CvfA protein has an RNA binding domain (KH domain), we hypothesized that CvfA hydrolyzes the 2',3'-cyclic phosphodiester linkage at the 3'-terminal of RNA. CvfA was incubated with RNA containing guanosine 2',3'-cyclic phosphate in its 3'-terminal nucleoside ([Np]$_p$G$>p^*$), and the products were digested with nuclease P1 and analyzed by cellulose TLC. In the presence of CvfA, the contents of pG$>p^*$ were decreased to 86% of the control (Fig. 5A). On the other hand, labeled free phosphate, which was produced by nuclease P1 from (Np)$_p$G$>p^*$, an estimated hydrolyzed product of [(Np)$_p$G$>p^*$, was increased. These results suggest that CvfA has phosphodiesterase activity against the 2',3'-cyclic nucleotide structure at the 3'-terminal of RNA. Scatchard plot analysis demonstrated that the $K_m$ value of the hydrolysis activity of CvfA against (Np)$_p$G$>p^*$ was 1.1 mM (Fig. 5B, Table 4).

**Phosphodiesterase Activity of CvfA Proteins with Mutation in the HD Domain**—To determine the CvfA structure required for the phosphodiesterase activity, we focused on the consensus residues of the HD domain (Fig. 1B, magenta and green). Among these, the residues corresponding to His$^{567}$ and Asp$^{368}$ were important for the phosphohydrolase activity of the HD domain–possessing enzymes, such as the RpfG family response regulator, tRNA-nucleotidyltransferase, and

### TABLE 4

| Substrates | $K_m$ $\mu$m | $V_{max}$ $\mu$mol/min/mg | $k_{cat}$ $s^{-1}$ |
|------------|--------------|----------------------------|-------------------|
| Bis-pNPP   | 30,000       | 0.75                       | 0.73              |
| 2',3'-cAMP | 17,000       | 0.93                       | 0.91              |
| 2',3'-cGMP | 15,000       | $1.5 \times 10^{-3}$      | $1.4 \times 10^{-3}$ |

**FIGURE 4.** CvfA phosphodiesterase activity with 2',3'-cyclic AMP and 2',3'-cyclic GMP as a substrate. A–C, CvfA protein (0.4 $\mu$g) was incubated with 20 mM 3',5'-cAMP or 2',3'-cAMP (A), 3',5'-cGMP or 2',3'-cGMP (B), and 2',3'-cCMP (C) in buffer containing 50 mM HEPES-KOH (pH 9.0) and 1 mM MnCl$_2$, and the reaction products were analyzed by cellulose TLC. The arrowhead indicates 3'-AMP or 3'-GMP as a hydrolyzed product. D and E, dependence on 2',3'-cAMP or 2',3'-cGMP concentration of CvfA phosphodiesterase activity was analyzed. The product (3'-AMP or 3'-GMP) was quantified by measuring the signal intensity using Image Gauge software.
phosphodiesterase type 5 (19, 25, 26). Regarding His367, Asp368, and other conserved residues among various pathogenic bacteria (His338, Lys371, and Asp423), we constructed amino acid-substituted CvfA proteins. They were expressed in *E. coli* and corresponding Fractions I and III of the mutants were prepared as well as wild-type CvfA. In Fractions I and III prepared from *E. coli* expressing these CvfA mutant proteins, the phosphodiesterase activity against bis-pNPP was lower than that in wild-type (Fraction I, data not shown; Fraction III, Fig. 6). The presence of the mutant CvfA proteins in these fractions was confirmed by SDS-PAGE (Fig. 6). These results suggest that the conserved residues in the HD domain of CvfA (His338, His367, Asp368, Lys371, and Asp423) are required for the phosphodiesterase activity.

**Hemolysin Production by the CvfA Mutant Proteins in *S. aureus* Cells**—To examine whether the phosphodiesterase activity of CvfA is required for the hemolysin production in *S. aureus* cells, we studied whether the expression of the CvfA mutant proteins can complement the decreased hemolysin production in the cvfA-deletion mutant. The expression of wild-type CvfA protein under the control of the IPTG inducible promoter restored hemolysin production in the cvfA-deletion mutant, MP1129, in an IPTG-dependent manner (data not shown). In contrast, the expression of CvfA mutant proteins (H338A, H367A, D368A, K371A, and D423A) did not restore hemolysin production in the M1129 strain. These results suggest that CvfA mutant proteins with decreased phos-

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**FIGURE 5.** *CvfA* phosphodiesterase activity against 2',3'-cyclic nucleotide structure at the 3'-terminal of RNA. A, CvfA protein (0.4 µg) was incubated with 200 nM (Np)_G>p* in the buffer containing 50 mM HEPES-KOH (pH 9.0) and 1 mM MnCl₂. p* means 32P-labeled phosphate. The products were digested by nuclease P1 and separated by TLC and analyzed by autoradiography. The black arrowhead indicates pG>p*, and the white arrowhead indicates free 32P-labeled phosphate. The signal intensity was indicated at the side of each signal. B, dependence on (Np)_G>p* concentration of CvfA phosphodiesterase activity was analyzed.

**FIGURE 6.** Phosphodiesterase activity of amino acid-substituted CvfA protein. Phosphodiesterase activity of a Tween 20-solubilized membrane (Fraction III) prepared from *E. coli* expressing mutated CvfA protein (H338A, H367A, D368A, K371A, and D423A) was measured using bis-pNPP as a substrate. The results of SDS-PAGE analysis of Fraction III protein were shown below the graph.

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phodiesterase activity are not functional for hemolysin production in S. aureus.

The RN4220 strain is mutagenized by nitrosoguanidine exposure (5, 28). There might be some uncharacterized mutations that affect the function of the cvfA gene product in hemolysin production. Therefore, we examined whether the CvfA mutant proteins complement decreased hemolysin production in the cvfA deletion mutant, in a non-mutagenized strain, NCTC8325-4. Disruption of the cvfA gene in NCTC8325-4 induced a decrease in hemolysin production (Fig. 7). In this cvfA-deletion mutant, the expression of wild-type CvfA protein restored hemolysin production in an IPTG-dependent manner (Fig. 7). In contrast, the expression of CvfA mutant proteins (H338A, H367A, D368A, K371A, and D423A) did not restore hemolysin production. These results suggest that CvfA mutant proteins with decreased phosphodiesterase activity are not functional for hemolysin production in both RN4220 and NCTC8325-4. Thus, phosphodiesterase activity of CvfA is required for hemolysin production in S. aureus cells.

**Complementation Activities by CvfA Mutant Proteins against the Attenuated Virulence in cvfA-deletion Mutant**—Next, we studied whether the expression of the CvfA mutant proteins can complement the attenuated virulence in the cvfA-deletion mutant using a silkworm-infection model. The expression of wild-type CvfA protein restored the silkworm killing ability of the cvfA-deletion mutant (Fig. 8). In contrast, the expression of CvfA mutant proteins (H338A, H367A, D368A, K371A, and D423A) did not restore the silkworm killing ability of the cvfA-deletion mutant (Fig. 8). In the absence of IPTG, more than 50 h was required for all the cvfA-deletion mutants possessing each expression vector to kill half of the silkworms (data not shown). These results suggest that the expression of the CvfA mutant proteins with decreased phosphodiesterase activity cannot complement the attenuated virulence in the cvfA-deletion mutant. Thus, phosphodiesterase activity of CvfA is required for virulence in S. aureus.

**DISCUSSION**

In this paper, we demonstrated that CvfA protein has phosphodiesterase activity. CvfA mutant proteins in which the consensus amino acid residues of the HD domain were substituted had reduced phosphodiesterase activity, and its expression in S. aureus cvfA-deletion mutant did not complement the decreased hemolysin production and the attenuated virulence against silkworms. Therefore, we concluded that phosphodiesterase activity of CvfA is required for virulence in S. aureus.

CvfA has phosphodiesterase activity against 2',3'-cAMP and 2',3'-cGMP. Although many organisms from prokaryotes to eukaryotes have some phosphodiesterases against the 2',3'-cyclic nucleotide, its physiological role is not well understood. The present study is the first report suggesting that phosphodiesterase against the 2',3'-cyclic nucleotide functions in bacterial virulence. The 2',3'-cyclic nucleotide is produced at the 3'-terminal of the RNA when cleaved by endonuclease. Many functional RNAs, such as tRNA and small RNA, are produced by the cleavage of pre-RNA. If the 2',3'-cyclic nucleotide structure at the 3'-end of RNA is not converted to the 3'-phosphate or 3'-OH form, the following RNA processing such as degradation or addition of nucleotides cannot occur (29–32). It is possible that the enzyme hydrolyzing the 2',3'-cyclic nucleotide acts on such RNA processing. For example, *E. coli* tRNA-nucleotidyltransferase, an enzyme possessing the HD domain, is assumed to process tRNA 3'-terminal by hydrolyzing the 2',3'-cyclic phosphodiester linkage (19). CvfA has an RNA binding domain (KH domain), and hydrolyzes the 2',3'-cyclic phosphodiester linkage at the 3'-terminal of Staphylococcal RNA. The $K_m$ value of the CvfA phosphodiesterase activity against $\text{NP}_{60}\text{G} > p$ was 1.1 $\mu M$, which was extremely low compared with that against the mononucleotide G.$> p$. From these results,
we assume that CvFA binds to a specific RNA via its KH domain, and hydrolyzes the 2′,3′-cyclic phosphodiester linkage at the 3′-terminal of the target RNA, and regulate virulence. Such a virulence regulatory system has not been previously described.

It is now evident that di-cGMP works as a second messenger in bacteria regulating many cellular functions, including virulence. In Salmonella enterica var. typhimurium and Vibrio cholerae, factors involved in di-cGMP hydrolysis are required for the expression of virulence factor and survival in mice (33–35). In Xanthomonas campestris, RpfG, an enzyme with the HD domain and hydrolysis activity against di-cGMP, is required for expressing the toxin (26). On the other hand, CvFA did not have hydrolysis activity against di-cGMP. Therefore, it is thought that the target molecule regulated by CvFA phosphodiesterase activity is not a di-cGMP. Further studies are needed to determine the target molecule hydrolyzed by CvFA.

CvFA has a transmembrane domain and its phosphodiesterase activity was recovered from the membrane fraction of E. coli expressing CvFA. In Bacillus subtilis, ymdA, a homologous gene of cvfA, is localized at the membrane based on analysis using green fluorescent protein fusion protein (36). There are some known systems by which membrane-bound factors convey the extracellular information to the intracellular signal network: the two-component system in which the signal is transmitted by phosphorylation of the transcription factor, and the εE system in which the signal is transmitted by degradation of proteins (9, 10, 37). Similar to these systems, CvFA might transmit the signal in response to the extracellular information and regulate virulence at the membrane. Elucidating the CvFA virulence regulatory system would contribute to the understanding of an important signaling pathway commonly utilized by many pathogenic bacteria.

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REFERENCES

1. Crossly, K. B., and Archer, G. L. (1997) The Staphylococci in Human Disease, Churchill Livingstone, New York
2. Lowy, F. D. (1998) N. Engl. J. Med. 339, 520–532
3. Dinges, M. M., Orwin, P. M., and Schlievert, P. M. (2000) Clin. Microbiol. Rev. 13, 16–34
4. Foster, T. J. (2005) Nat. Rev. Microbiol. 3, 948–958
5. Peng, H. L., Novick, R. P., Kreiswirth, B., and Schlievert, P. M. (1988) J. Bacteriol. 170, 4365–4372
6. Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993) EMBO J. 12, 3967–3975
7. Cheung, A. L., Koomen, J. M., Butler, C. A., Projan, S. J., and Fischetti, V. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6462–6466
8. Giraud, A. T., Cheung, A. L., and Nagel, R. (1997) Arch. Microbiol. 168, 53–58
9. Yarwood, J. M., McCormick, J. K., and Schlievert, P. M. (2001) J. Bacteriol. 183, 1113–1123
10. Fournier, B., Klier, A., and Rapoport, G. (2001) Mol. Microbiol. 41, 247–261
11. Novick, R. P. (2003) Mol. Microbiol. 48, 1429–1449
12. Kaito, C., Akimitsu, N., Watanabe, H., and Sekimizu, K. (2002) Microb. Pathog. 32, 183–190
13. Kaito, C., Kurokawa, K., Matsumoto, Y., Terao, Y., Kawabata, S., Hamada, S., and Sekimizu, K. (2005) Mol. Microbiol. 56, 934–944
14. Matsumoto, Y., Kaito, C., Morishita, D., Kurokawa, K., and Sekimizu, K. (2007) Infect. Immun. 75, 1964–1972
15. Kaito, C., Morishita, D., Matsumoto, Y., Kurokawa, K., and Sekimizu, K. (2006) Mol. Microbiol. 62, 1601–1617
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Inoue, R., Kaito, C., Tanabe, M., Kamura, K., Akimitsu, N., and Sekimizu, K. (2001) Mol. Genet. Genomics 266, 564–571
18. Li, S., and Wilkinson, M. F. (1997) BioTechniques 23, 588–590
19. Yakunin, A. F., Proudfoot, M., Kuznetsova, E., Savchenko, A., Brown, G., Arrowsmith, C. H., and Edwards, A. M. (2004) J. Biol. Chem. 279, 36819–36827
20. Tyc, K., Kellenberger, C., and Filipowicz, W. (1987) J. Biol. Chem. 262, 12994–13000
21. Fabret, C., Ehrlich, S. D., and Noirot, P. (2002) Mol. Microbiol. 46, 25–36
22. Novick, R. P. (1991) Methods Enzymol. 204, 587–636
23. Vandenesch, F., Kornblum, J., and Novick, R. P. (1991) J. Bacteriol. 173, 6313–6320
24. Aravind, L., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 469–472
25. Turko, I. V., Francis, S. H., and Corbin, J. D. (1998) J. Biol. Chem. 273, 6460–6466
26. Ryan, R. P., Fouhy, Y., Lacey, J. F., Crossman, L. C., Spore, H., Ye, W. Y., Zhang, L. H., Heeb, S., Camara, M., Williams, P., and Dow, J. M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6712–6717
27. Romling, U., Gomelsky, M., and Galperin, M. Y. (2005) Mol. Microbiol. 57, 629–639
28. Traber, K., and Novick, R. (2006) Mol. Microbiol. 59, 1519–1530
29. Li, Z., Pandit, S., and Deutscher, M. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2856–2861
30. Kennell, D. (2002) J. Bacteriol. 184, 4645–4657
31. Gonzalez, T. N., Sidrauski, C., Dorfler, S., and Walter, P. (1999) EMBO J. 18, 3119–3132
32. Trulzsch, K., Roggenkamp, A., Pelludat, C., Rakin, A., Jacobi, C., and Heesemann, J. (2001) Microbiology 147, 203–213
33. Hisert, K. B., MacCoss, M., Shiloh, M. U., Darwin, K. H., Singh, S., Jones, R. A., Eht, S., Zhang, Z., Gaffney, B. L., Gandotra, S., Holden, D. W., Murray, D., and Nathan, C. (2005) Mol. Microbiol. 56, 1234–1245
34. Tischler, A. D., and Camilli, A. (2005) Infect. Immun. 73, 5873–5882
35. Tamayo, R., Tischler, A. D., and Camilli, A. (2005) J. Biol. Chem. 280, 33324–33330
36. Hunt, A., Rawlins, J. P., Thomaides, H. B., and Errington, J. (2006) Microbiology 152, 2895–2907
37. Ades, S. E. (2004) Curr. Opin. Microbiol. 7, 157–162
38. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani, U., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., and Hiramatsu, K. (2001) Lancet 357, 1225–1240
39. Novick, R. (1967) Virolology 33, 155–166
40. Rao, L., LeBlanc, D. J., and Ferretti, J. J. (1992) Gene (Amst.) 120, 105–110
41. Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998) Microbiology 144, 3097–3104