CvfA Protein and Polynucleotide Phosphorylase Act in an Opposing Manner to Regulate Staphylococcus aureus Virulence*5

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Background: Production of 3′-phosphorylated RNA by CvfA affects S. aureus virulence gene expression.

Results: Disrupting pnpA-encoding exonuclease suppressed the cvfA-deleted mutant phenotype. Purified PNase did not degrade 3′-phosphorylated RNA.

Conclusion: CvfA-produced 3′-phosphorylated RNA inhibits PNase-induced RNA degradation, resulting in hemolysin production by S. aureus.

Significance: Altering the nucleotide structure at the RNA 3′ terminus regulates S. aureus virulence.

We previously identified CvfA (SA1129) as a Staphylococcus aureus virulence factor using a silkworm infection model. S. aureus cvfA-deleted mutants exhibit decreased expression of the agr locus encoding a positive regulator of hemolysin genes and decreased hemolysin production. CvfA protein hydrolyzes a 2′,3′-cyclic phosphodiester bond at the RNA 3′ terminus, producing RNA with a 3′-phosphate (3′-phosphorylated RNA, RNA with a 3′-phosphate). Here, we report that the cvfA-deleted mutant phenotype (decreased agr expression and hemolysin production) was suppressed by disrupting pnpA-encoding polynucleotide phosphorylase (PNase) with 3′- to 5′-exonuclease activity. The suppression was blocked by introducing a pnpA-encoding PNase with exonuclease activity but not by a pnpA-encoding mutant PNase without exonuclease activity. Therefore, loss of PNase exonuclease activity suppressed the cvfA-deleted mutant phenotype. Purified PNase efficiently degraded RNA with 2′,3′-cyclic phosphate at the 3′ terminus (2′,3′-cyclic RNA), but it inefficiently degraded 3′-phosphorylated RNA. These findings indicate that 3′-phosphorylated RNA production from 2′,3′-cyclic RNA by CvfA prevents RNA degradation by PNase and contributes to the expression of agr and hemolysin genes. We speculate that in the cvfA-deleted mutant, 2′,3′-cyclic RNA is not converted to the 3′-phosphorylated form and is efficiently degraded by PNase, resulting in the loss of RNA essential for expressing agr and hemolysin genes, whereas in the cvfA/pnpA double-disrupted mutant, 2′,3′-cyclic RNA is not degraded by PNase, leading to hemolysin production. These findings suggest that CvfA and PNase competitively regulate RNA degradation essential for S. aureus virulence.

Regulation of RNA stability is important for cells to rapidly adapt to extracellular environmental changes. In bacteria, nuclease complexes called degradosomes degrade mRNA (1). Bacillus subtilis degradosomes contain RNase Y with endonuclease activity (2), PNase2 with 3′- to 5′-exonuclease activity (3), and other nucleases. The rny (ymdA) gene encoding RNase Y is essential for cell growth in B. subtilis (4). The gapA operon mRNA encoding a glycolytic enzyme, S-adenosylmethionine-dependent riboswitch RNA, and rpsO mRNA encoding ribosomal protein S15 are substrates of RNase Y (2, 5, 6). These substrates are thought to be endonucleolytically cleaved by RNase Y and subsequently degraded by other nucleases. PNase is conserved among bacteria, plant, and metazoa (7). Deletion mutants of pnpA-encoding PNase in B. subtilis and Escherichia coli can grow at 37 °C, but they have a cold-sensitive phenotype (8–12). Deletion mutants of pnpA in Salmonella enterica and Yersinia spp. show increased expression of virulence genes (13–15), indicating that the pnpA gene product negatively regulates bacterial virulence. The molecular mechanism underlying the effect of pnpA on the expression of specific genes is not known.

We previously identified novel virulence genes of S. aureus using a silkworm infection model (16, 17). cvfA is a virulence gene, and its gene product is a component of S. aureus degradosomes (18, 19). CvfA is a homolog of B. subtilis RNase Y. Unlike the B. subtilis rny deletion mutant, the S. aureus cvfA-deleted mutant is viable and grows normally in nutrient medium (17). The cvfA-deleted mutant of S. aureus exhibits decreased expression of the agr locus encoding virulence regulators and decreased hemolysin production (17). Furthermore, the cvfA-deleted mutant of S. aureus and Streptococcus pyogenes has attenuated virulence in mice and silkworms (17). In S. pyogenes, deletion of cvfA affects the expression of 30% of all genes, including virulence genes and metabolic genes (20, 21). We previously demonstrated that CvfA cleaves the 2′,3′-cyclic phosphodiester linkage of the 3′ terminus of RNA and pro-

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This article contains Tables S1 and S2.

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2 The abbreviations used are: PNase, polynucleotide phosphorylase; MRSA, methicillin-resistant S. aureus; 2′,3′-cyclic RNA, 2′,3′-cyclic phosphate at the 3′ terminus; 3′-phosphorylated RNA, RNA with a 3′-phosphate; 3′-OH RNA, 3′-hydroxylated RNA; Ni-NTA, nickel-nitriol triacetic acid.
duces 3’-monophosphorylated RNA (22). It remained unclear, however, how modification of the 3’-terminal structure of RNA by CvfA affects the expression of S. aureus virulence genes.

In this study, we searched for a gene that genetically interacts with cvfA to reveal the molecular mechanism of virulence gene regulation by CvfA. Our findings revealed that disruption of pnpA encoding PNPase with exonuclease activity suppressed the phenotype of the cvfA-deleted mutant. Furthermore, RNA degradation activity of PNPase was affected by the structure of the 3’-terminal nucleotide of the RNA substrate.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—The E. coli JM109 strain was used to host pET-11a, pND50, and their derivatives. E. coli strains transformed with the plasmids were aerobically cultured in the presence of 100 μg/ml ampicillin or 25 μg/ml chloramphenicol. S. aureus strains were aerobically cultured in tryptic soy broth at 37 °C. To transform the S. aureus strain with plasmids, 10 μg/ml chloramphenicol was added to tryptic soy broth. To transform the plasmids, 10 μg/ml phleomycin, or 25 μg/ml erythromycin, or 20 μg/ml ampicillin was added.

**DNA Manipulation**—Transformation of E. coli, extraction of plasmid DNA, and polymerase chain reaction (PCR) were performed according to Sambrook et al. (23). S. aureus was transformed using electroporation (24). Introduction of point mutations into plasmid DNA was performed according to Li et al. (25).

**Construction of the pnpA-disrupted Mutant and Plasmids Carrying Mutated pnpA Genes**—DNA fragments containing the internal region (+84 to +584 bp) of pnpA (+1 as the first nucleotide of the open reading frame) was amplified by PCR using oligonucleotide primers (Table 2) and genome DNA of NCTC8325-4 strain as the template. The amplified fragment was inserted into EcoRI and BamHI sites of pMutinT3, resulting in a targeting plasmid. S. aureus RN4220 strain was electroporated with the targeting plasmid, resulting in a strain resistant to erythromycin. The plasmid that was integrated into the pnpA gene was transferred to the NCTC8325-4 strain using phage 80a, which resulted in the pnpA-disrupted mutant. Disruption of pnpA in NCTC8325-4 strain was confirmed by Southern blot analysis.

The DNA fragment containing intact pnpA gene was amplified by PCR using oligonucleotide primers (Table 2) and genome DNA of NCTC8325-4 strain as the template. The amplified DNA fragment was inserted into EcoRI and BamHI sites of pMutinT3, resulting in a targeting plasmid. S. aureus RN4220 strain was electroporated with the targeting plasmid, resulting in a strain resistant to erythromycin. The plasmid that was integrated into the pnpA gene was transferred to the NCTC8325-4 strain using phage 80a, which resulted in the pnpA-disrupted mutant. Disruption of pnpA in NCTC8325-4 strain was confirmed by Southern blot analysis.

**Measurement of Hemolysin Production**—S. aureus overnight culture (2 μl) was spotted onto tryptic soy agar plates containing 5% sheep erythrocytes and incubated for 12 h at 37 °C. The clear zone around the S. aureus colony was evaluated.
TABLE 2

Primers used in this study

| Target          | Primer 1   | Primer 2   | Sequence (5′−3′) |
|-----------------|------------|------------|-----------------|
| pnpA disruption | F-EcoRI-1117 | R-EcoRI-1117 | CCGAATTTCAGGCTCATGATAGTTGCC |
| pnpA complementation | F-BamHI pnpA-c     | R-EcoRI pnpA-c   | AATGATCTAGATGACCTCTCTAACACAT |
| Mutated pnpA    | F-R402A/R403A   | R-R413D     | CCAATATCTCATCATGTCCGCTGAGTTAG |
|                 | F-H407D       | R-D496G     | TCGTCTGGATCATGACGACGACGGAT |
| PnpA overproduction | F-D96G        | R-D496G     | CAGGAGACCTTTAAAAACCCATATACTGACGTAC |
|                 | F-R-N-His-pnpA-op      | R-N-His-pnpA-op   | CGCTGAGAATATTTTTGTTTTTTAAATGGAGAGATATATGACGACGACGACGACGACGAC |
| saeP (qRT-PCR)  | Frt-saeP      | Ret-saeP    | CAAATTTGAAAGAATGAGATTA |
| saeQ (qRT-PCR)  | Frt-saeQ      | Ret-saeQ    | GAAATTTACAGCGCGGATTT |
| adhE (qRT-PCR)  | Frt-adhE      | Ret-adhE    | CAAAGAACGCGACGACGGCTCAT |

The role of nucleotide structure at 3′-terminus of RNA

Reporter Assay—S. aureus strains were transformed with reporter plasmids carrying the agr P2, agr P3, and hla promoter. Overnight cultures of the transformed strains were inoculated to 100-fold amounts of fresh tryptic soy broth and aerobically cultured at 37 °C. The cultivated cells were collected by centrifugation and lysed in a buffer (20 mM KH$_2$PO$_4$ (pH 7.8), 0.04% Triton X-100, 0.1 mM DTT, 10 μg/ml lysozyme, 1 tablet of protease inhibitor (Roche Applied Science)). Cell lysate supernatant was incubated with luciferase substrate, and luminescence was measured using a luminometer (Berthold Technologies, Bad Wildbad, Germany). The promoter activity was calculated as luminescence units/mg of protein.

Microarray Analysis—Overnight cultures of S. aureus strains were inoculated to 100-fold amounts of fresh tryptic soy broth and aerobically cultured to A$_{600}$ = 4 at 37 °C. The cultured cells were collected by centrifugation and treated with RNAProtect Bacteria Reagent (Qiagen). The cells were washed with phosphate-buffered saline and lysed by lysozyme. Total RNA was extracted using an RNeasy mini kit according to the manufacturer’s protocol (Qiagen), and any remaining DNA was degraded with RNase-free DNase (Promega). cDNA was synthesized from the RNA using Multiscribe Reverse Transcriptase (Applied Biosystems). Quantitative real time PCR was performed using cDNA as a template, SYBR Premix ExTaq (Takara Bio), and primers (Table 2). The signals were detected using an ABI PRISM 7700 sequence detector (Applied Biosystems).

Purification of PNPase—S. aureus pnpA gene fused with His$_6$ tag was inserted into pET11a, resulting in pNHis-pnpA. E. coli BL21(DE3) harboring pLysS was transformed with pN-His-pnpA. The transformed strain was aerobically cultured in 100 ml of Luria-Bertani broth at 37 °C to A$_{600}$ = 0.5; 1 mM isopropyl β-d-1-thiogalactopyranoside was added, and the mixture was cultured overnight at 16 °C. The cells were collected by centrifugation and lysed by freezing and thawing, followed by sonication. The sample was centrifuged, and ammonium sulfate was added to the supernatant at a final concentration of 100%. The resulting precipitate was dissolved in buffer A (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20% glycerol, 1 mM imidazole) and subjected to a Ni-NTA column (ProBond Resin, Invitrogen). The column was washed several times with buffer B (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20% glycerol, 67 mM imidazole), and the proteins were eluted with buffer C (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20% glycerol, 1 mM imidazole). The amount of protein in each fraction was determined by the Bradford assay. To obtain an antibody against PNPase, purified PNPase (0.2 mg) was subcutaneously injected into a Japanese white rabbit five times at 2-week intervals. Blood was collected from the rabbit and used for IgG purification by protein G-Sepharose.

Measurement of Poly(A) Polymerization Activity and Phosphorylolytic Activity—To determine poly(A) polymerization activity, PNPase protein was mixed with 5 mM ADP containing 0.3 μM [2,8-3H]ADP in a reaction buffer (50 mM Tris-HCl (pH 8.0), 5 mM MgCl$_2$, 5 mM ADP) and incubated at 37 °C for 15 min. The reaction was terminated by adding 0.1% perchloric acid, mixed with the same volume of 10% TCA, and incubated on ice for 10 min. The precipitated poly(A) was trapped by a glass filter (Whatman), and the radioactivity on the filter was measured by a scintillation counter (LC 5000TS, Beckman).
To determine the phosphorolytic activity, PNPase protein was mixed with 30 \( \mu \)g poly(A) (Sigma) in a reaction buffer (50 mM Tris-HCl (pH 8.4), 5 mM MgCl\(_2\), 60 mM KCl, 10 mM sodium phosphate) at 37 °C for 10 min. The reaction mixture was mixed with a 2.5 volume of ethanol and centrifuged. The A\(_{260}\) of the supernatant was measured to calculate the amount of released ribonucleoside diphosphates.

**Phosphorolytic Activity of PNPase against Different RNA Substrates**—RNA substrates (5’-AAAAAAGG-3’) with different 3’-terminal nucleotides were synthesized using the phosphoramidite method. The 3’-hydroxylated RNA and 3’-phosphorylated RNA were obtained from Hokkaido System Science, Sapporo, Japan. The 2’,3’-cyclic RNA was obtained from GeneDesign, Osaka, Japan. Structures of RNA substrates were confirmed by electrospray ionization time-of-flight mass spectrometry (microTOF, Bruker Daltonics). PNPase protein was mixed with 30 \( \mu \)g RNA substrate in a reaction buffer (50 mM Tris-HCl (pH 8.4), 5 mM MgCl\(_2\), 60 mM KCl, 10 mM sodium phosphate) at 37 °C for 10 min. The reaction was terminated by the addition of 2 \( \times \) loading buffer (95% formamide, 0.025% SDS, 18 mM EDTA, 0.025% xylencyanol, 0.025% bromphenol blue) and boiling. The RNA sample was electrophoresed in a 7 M urea, 20% polyacrylamide gel and stained with SYBR Green. Images were analyzed using an image analyzer (Typhoon FLA9000, GE Healthcare). To measure the amount of the RNA degradation product, PNPase protein was mixed with different amounts of RNA substrate in the reaction buffer at 37 °C for 10 min. The reaction mixture was then mixed with 2.5 volumes of ethanol and centrifuged. The A\(_{260}\) of the supernatant was measured to calculate the amount of released ribonucleoside diphosphates. The \( K_m \) and \( V_{\text{max}} \) values of PNPase protein against different RNA substrates were determined by nonlinear regression analysis using Graph Pad Prism version 5.0c.

**Detection of PNPase and CvFA**—-*S. aureus* cells were collected, and the cell walls were lysed in digestion buffer (30% raffinose, 50 mM Tris-HCl (pH 7.5), 145 mM NaCl, 100 \( \mu \)g/ml lysostaphin, 10 units/ml DNase I) at 37 °C for 30 min. The cells were collected by centrifugation and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 units/ml DNase I). The protein concentration was determined by the Bradford assay. The protein was electrophoresed in 15% SDS-polyacrylamide gels. The proteins were blotted to a PVDF membrane (Immobilon-P, Merck). The membrane was treated with blocking buffer (TBST: 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20, 5% Easy Blocker (GeneTex, Irvine, CA)) at room temperature for 1 h.

The membrane was treated with blocking buffer containing 1:1000 anti-PnpA IgG or anti-CvFA IgY (22) at room temperature for 1 h. After washing with TBST, the membrane was treated with a blocking buffer containing 1:200 anti-rabbit IgG conjugated with alkaline phosphatase or anti-chicken IgY conjugated with HRP at room temperature for 1 h. After washing with TBST, the membrane for detecting PNPase was reacted with a substrate for alkaline phosphatase (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Roche Applied Science). The membrane for detecting CvFA was reacted with an HRP substrate (Western Lightning Plus ECL, PerkinElmer Life Sciences) and subsequently exposed to film (Hyperfilm ECL, GE Healthcare).

**Role of Nucleotide Structure at 3’ Terminus of RNA**

**Silkworm Infection Experiment**—The infection experiment using silkworms was performed according to the previously described method (26). Fertilized eggs were purchased from Ehime Sansyu (Ehime, Japan). Hatched larvae were raised to fifth instar larvae by feeding an artificial diet. *S. aureus* overnight cultures were centrifuged, and the cells were suspended in saline. 2-Fold serial diluted bacterial solutions were injected into the hemolymph of silkworms (n = 10). Surviving silkworms were counted at 24 h after the injection. LD\(_{50}\) values were determined from dose-survival curves.

**RESULTS**

**Decreased Hemolysin Production and Agr Expression in the cvfA Deletion Mutant Is Suppressed by the Disruption of pnpA**—To investigate the molecular mechanisms underlying the regulation of hemolysin production by CvFA, we searched for gene mutations that suppress the decreased hemolysin production of the *cvfA*-deleted mutant. Because CvFA has phosphodiesterase activity against 2’,3’-cyclic phosphodiester linkage at the 3’-terminal nucleotide of RNA, we hypothesized that modification of the RNA by CvFA would affect the sensitivity of the RNA against other RNA metabolic enzymes. To examine this possibility, we constructed double-disrupted mutants of cvfA and eight other genes encoding RNases, including RNase III (SA1076), SA0489, RNase HII (SA1087), SA1335, SA0450, PNPase (SA1117), RNase R (SA0735), and YhaM (SA1660), and examined their hemolysin production. Disruption of SA1076, SA1335, or SA1660 in the *cvfA*-deleted mutant caused slow growth or was not stable and thus not further evaluated. Disruption of SA0449 or SA0735 in the *cvfA*-deleted mutant was not successful. Disruption of SA0450 or SA1087 in the *cvfA*-deleted mutant had no effect on hemolysin production. *cvfA*-deleted mutants with disruption of *pnpA*-encoding PNPase produced greater amounts of hemolysins than *cvfA*-deleted mutants without disruption, which normally produce only small amounts of hemolysins (Fig. 1A). Thus, disruption of *pnpA* suppressed the decreased hemolysin production of *cvfA*-deleted mutants. The doubling speed of the *cvfA/pnpA* double-disrupted mutant was indistinguishable from that of the parent strain and the *cvfA*-deleted mutant (Fig. 1B). In addition, the promoter activity of the *hla* gene encoding \( \alpha \)-hemolysin in the *cvfA/pnpA* double-disrupted mutant was higher than that in the *cvfA*-deletion mutant (Fig. 1C). In the *cvfA/pnpA* double-disrupted mutant, promoter activities of P2 and P3 of the *agr* locus, which positively regulate *hla* transcription, were higher than those in the *cvfA*-deleted mutant (Fig. 1, D and E). Therefore, we concluded that the decreased hemolysin production and *agr* expression in the *cvfA*-deleted mutant were suppressed by the disruption of *pnpA* and that *cvfA* genetically interacts with *pnpA*.
The deletion of cvfA affects the expression of various genes in S. aureus (27) and S. pyogenes (20). We examined whether the disruption of pnpA restores the effects of cvfA deletion on gene expression other than agr and hla genes. First, we performed microarray analysis of the cvfA-deleted mutant. The cvfA deletion affected the expression of 20% of S. aureus genes (supplemental Tables S1 and S2), consistent with previous reports (20, 27). Among the genes with altered expression, we focused on the decreased expression of saeP, which is encoded by the sae locus that positively regulates S. aureus hemolysin production (28). The sae locus contains four genes, saeP, saeQ, saeR, and saeS. The saeP and saeQ genes are transcribed as a saeP-saeQ-
saeR-saeS polycistron and are required for saeRS function (29). It was recently suggested that the cvfA gene affects the processing of the saePQRS transcript (27). Quantitative reverse transcriptase (RT)-PCR analysis confirmed that the expressions of saeP and saeQ were decreased compared with the parent strain (Fig. 1, F and G). The expression of saeP and saeQ was higher in the cvfA/pnpA double-disrupted mutant than in the cvfA-deleted mutant (Fig. 1, F and G). In addition, in the cvfA-deleted mutant, the expression of various metabolic genes involved in amino acid biosynthesis, acetate metabolism in glycolysis, citric acid cycles, and nutrient transporter genes were altered (supplemental Tables S1 and S2). Quantitative RT-PCR analysis confirmed that expression of the adhE gene, which is a metabolic gene involved in acetate metabolism, was decreased in the cvfA-deleted mutant compared with the parent strain (Fig. 1H). The expression of adhE was higher in the cvfA/pnpA double-disrupted mutant than in the cvfA-deleted mutant. Therefore, the altered expression of saeP, saeQ, and adhE genes in the cvfA-deleted mutant was suppressed by the disruption of pnpA.

We further examined whether the decreased virulence of the cvfA-deleted mutant against animals was blocked by the disruption of pnpA. The LD50 value of the cvfA-deleted mutant against silkworms or mice was larger than that of the parent strain (Table 3). The LD50 value of cvfA/pnpA double-disrupted mutant against silkworms or mice was almost the same as that of the cvfA-deleted mutant. Thus, the decreased virulence of the cvfA-deleted mutant against animals was not attenuated by disrupting pnpA. These findings suggest that the phenotype of the cvfA-deleted mutant was not totally suppressed by the disruption of pnpA.

To evaluate the role of pnpA in S. aureus virulence, we examined the virulence property of a single mutant of pnpA. Hemoly-

### TABLE 3
Animal-killing ability of the S. aureus cvfA/pnpA double-disrupted mutant

| Strain | Silkworm LD50 | Mouse LD50 |
|--------|---------------|------------|
|        | (×10^7 cfu)   | (-Fold)    |
|        |               | (×10^7 cfu)| (-Fold)    |
| NCTC8325-4 (Parent) | 1.5 | 1.0 | 2.2 | 1.0 |
| CKP1129 (ΔcvfA) | >5.6 | >3.7 | 5.6 | 2.5 |
| M1117NC (ΔpnpA) | 0.58 | 0.39 | 1.8 | 0.81 |
| DM1NC (ΔcvfA/ΔpnpA) | >6.2 | >4.1 | 5.2 | 2.4 |

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### FIGURE 2

**Purification of S. aureus wild-type PNPase and mutated PNPases.**

A, domains of PNPase are schematically presented. PNPase contains two catalytic domains named PH-1 and PH-2 (58) and two RNA binding domains (59, 60). B, S. aureus PNPase fused with His₆ was overproduced in E. coli and purified by ammonium sulfate precipitation and Ni-NTA column chromatography. Fractions of ammonium sulfate precipitation (29/20g) and Ni-NTA column chromatography (5/15g) were analyzed by SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue. Poly(A) polymerization activity of each fraction is presented in Table 4. C, poly(A) polymerization activity of purified PNPase was measured at 37°C for 15 min using ADP as a substrate. Vertical axis represents the amount of ADP incorporated into poly(A), and horizontal axis represents the amount of added PNPase protein. D, mutated PNPases were purified by the same method for wild-type PNPase. Purified proteins (1 μg) were analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. E, poly(A) polymerization activities of mutated PNPases were measured using the same method as for wild-type PNPase. F, phosphorolytic activities of wild-type PNPase and mutated PNPases were measured at 37°C using poly(A) as a substrate.
sin production was increased in the \( pnpA \)-disrupted mutant compared with the parent strain (Fig. 1I). The increase in the hemolysin production was blocked by the introduction of a plasmid carrying intact \( pnpA \) (ppnpA; Fig. 1I). Thus, \( pnpA \) negatively affects hemolysin production. Furthermore, the \( LD_{50} \) value of the \( pnpA \) mutant against silkworms was smaller than that of the parent strain (Table 3). The \( LD_{50} \) value of the \( pnpA \) mutant against mice was slightly smaller than that of the parent strain (Table 3). Thus, the \( pnpA \) gene has a negative role in \( S. aureus \) virulence.

Mutated PNPase without RNA Degradation Activity Loses Complementation Activity against the \( cvfA/pnpA \) Double-disrupted Mutant—We constructed mutated PNPase proteins that lack phosphorolytic activity to determine whether the activity is required for the complementation activity of the increased hemolysin production of the \( cvfA/pnpA \) double-disrupted mutant. PNPase has two catalytic domains called PH-1 and PH-2 at the N-terminal region (Fig. 2A). Analysis of PNPase of \( Streptomyces antibioticus \) revealed that amino acid substitutions in PH-1 and PH-2 lead to the loss of the phosphorolytic activity of PNPase (30). We constructed \( E. coli \) strains overproducing mutated PNPase proteins with substitutions in amino acids that are conserved among bacteria (D96G, R402A/R403A, H407D, R413D, and D496G) and examined whether these mutated PNPases lose phosphorolytic activity. First, we purified wild-type recombinant PNPase protein from an \( E. coli \) strain expressing His\(_6\)-tagged PNPase by ammonium sulfate precipitation and Ni-NTA resin column chromatography (Fig. 2B). The enzyme activity of the wild-type PNPase was measured by poly(A) polymerization, which is a reverse reaction of phosphorolysis (Fig. 2C (3)). The specific activity of the eluted fraction from the Ni-NTA column was 46 \( \mu \)mol/15 min/mg of protein, which was 10 times higher than that of the ammonium sulfate precipitate fraction, and the recovery of activity was 33% (Table 4). Analysis of SDS-PAGE revealed that the purity of the final protein sample was greater than 90% (Fig. 2B). Furthermore, we purified the mutated PNPase by the same method for wild-type PNPase (Fig. 2D). The mutated PNPase R402A/R403A, H407D, and D496G did not show poly(A) polymerization activity, whereas D96G and R413D showed 10% poly(A) polymerization activity compared with wild-type PNPase (Fig. 2E). Amino acid substitution in enzymes may have different effects on forward and reverse reactions (30). We measured phosphorolytic activity of wild-type and mutated PNPases. The mutated PNPase R402A/R403A, H407D, and D496G showed a loss of phosphorolytic activity, whereas D96G and R413D showed 10% phosphorolytic activity compared with wild-type PNPase (Fig. 2F). Thus, R402A/R403A, H407D, and D496G lost both poly(A) polymerization activity and RNA degradation activity, whereas D96G and R413D retained both activities.

We then transformed the \( cvfA/pnpA \) double-disrupted mutant with plasmids expressing wild-type PNPase or mutated

| TABLE 4 | Purification of recombinant His-tagged PNPase |
|---------|---------------------------------------------|
| Fraction | Protein | Total activity | Specific activity | Yield | Purification |
|         | mg | \( \mu \)mol/15 min | \( \mu \)mol/15 min/mg | % | -fold |
| Ammonium sulfate | 14.3 | 69 | 4.8 | 100 | 1 |
| Nickel column | 0.5 | 23 | 46 | 33 | 10 |

FIGURE 3. Complementation activities of mutated PNPases against hemolysin production in the \( cvfA/pnpA \) double-disrupted mutant. A, overnight cultures of the \( S. aureus \) parent strain (NCTC8325-4), the \( cvfA \)-deleted mutant (CKP1129), and the \( cvfA/pnpA \) double-disrupted mutants (DM1NC), which were transformed with an empty plasmid (pND50), plasmid carrying wild-type \( pnpA \) (ppnpA), and plasmids carrying mutated \( pnpA \) (pD96G, pR402A/R403A, pH407D, pR413D, pD496G, and p\( \Delta \)RBD) were spotted onto nutrient agar plates containing 5% sheep erythrocytes and were incubated overnight at 37 °C. The clear zone around the colony reflects hemolysin activity. B, overnight cultures of \( S. aureus \) cells used in A were collected. The cell lysates were electrophoresed in SDS-polyacrylamide gel and subjected to Western blot analysis using anti-PNPase antibodies.
PNPases. The *cvfA/pnpA* mutant transformed with plasmid expressing wild-type PNPase (*ppnpA*) decreased hemolysin production compared with the *cvfA/pnpA* mutant transformed with an empty vector pND50 (Fig. 3A). In contrast, the *cvfA/pnpA* mutant transformed with plasmids expressing R402A/R403A, H407D, and D496G, which showed a loss of RNA deg-

**FIGURE 4.** PNPase does not degrade 3'-phosphorylated RNA. A–C, RNA substrates (5'-AAAAAAG-3') with different 3'-terminal nucleotides were synthesized using the phosphoramidite method. Structures of RNA substrates were confirmed by electrospray ionization time-of-flight mass spectrometry. D, PNPase (0, 13, 25, 50, 100, or 200 nM) was added to 30 μM 3'-OH RNA, 2',3'-cyclic RNA, or 3'-phosphorylated RNA and incubated for 10 min at 37 °C. The reaction product was electrophoresed in 7 M urea, 20% polyacrylamide gel, and the RNA was stained with SYBR Green. E–G, PNPase (50 nM) was added to 3'-OH RNA (0–32 μM) (E), 2',3'-cyclic RNA (0–500 μM) (F), or 3'-phosphorylated RNA (0–500 μM) (G) and incubated for 10 min at 37 °C. After the reaction, nondigested RNA was precipitated by ethanol, and the amount of ribonucleoside diphosphates in the centrifuged supernatant was calculated by measuring the A$_{260}$. 

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radiation activity, produced almost the same amount of hemolysins as the cvfA/pnpA mutant transformed with an empty vector pND50 (Fig. 3A). The cvfA/pnpA mutant transformed with plasmids expressing D96G and R413D, which retained RNA degradation activity, produced a smaller amount of hemolysins than the mutant transformed with an empty vector, and the production level was almost same as that of the mutant transformed with pnpA expressing wild-type PNPase (Fig. 3A). We performed a Western blotting analysis to measure the expression of PNPase in the cvfA/pnpA mutant. Each mutated PNPase other than R413D was expressed at either an equal or greater level as wild-type PNPase in the cvfA/pnpA mutant (Fig. 3B). The R413D mutant PNPase expression was lower than wild-type PNPase in the cvfA/pnpA mutant (Fig. 3B). Thus, loss of complementation activities of R402A/R403A, H407D, and D496G in the cvfA/pnpA mutant is due to the loss of enzymatic activity and not to the expression level of the mutated PNPases. These results suggest that that RNA degradation activity of PNPase protein is necessary for its complementation activity for the phenotype of the cvfA/pnpA mutant.

Mutated PNPase without the RNA Binding Domain Loses Complementation Activity against the cvfA/pnpA Double-disrupted Mutant—PNPase carries two RNA binding domains (residues 623–690) and obtained PNPase RBD in greater than 95% purity (Fig. 2D). The PNPase RBD retained poly(A) polymerization activity and phosphorolytic activity as wild-type PNPase (30). In contrast, the RNA binding domains were required for E. coli cell growth at low temperature. Crystal structural analysis indicated that the RNA binding domains are involved in the trimer formation of PNPase and accelerate the acquisition of substrate RNA (31, 32). We examined whether the RNA binding domains of PNPase are required for complementation of the phenotype of the cvfA/pnpA mutant. First, we constructed an E. coli strain overproducing mutated PNPase without the RNA binding domain (residues 623–690) and obtained PNPase ∆RBD in greater than 95% purity (Fig. 2D). The PNPase ∆RBD retained poly(A) polymerization activity and phosphorolytic activity (Fig. 2, E and F). We then transformed the cvfA/pnpA mutant with a plasmid expressing PNPase ∆RBD and examined hemolysin production. The transformed strain expressed PNPase ∆RBD and produced the same levels of hemolysins as the cvfA/pnpA mutant transformed with an empty vector (Fig. 3, A and B). Thus, although PNPase ∆RBD retained RNA degradation activity, it lost complementation activity against the phenotype of the cvfA/pnpA mutant. These results suggest that the RNA binding domain of PNPase is required for complementation of the cvfA/pnpA mutant phenotype.

3′-Phosphorylated RNA Is Resistant to Degradation by PNPase—Because Cvfa cleaves the 2′,3′-cyclic phosphodiester linkage of 2′,3′-cyclic RNA and produces 3′-phosphorylated RNA (22), PNPase degrades RNA from the 3′ terminus in the 5′ direction (3). As revealed above, because the phenotype of the cvfA-deleted mutant was suppressed by the disruption of pnpA-encoding PNPase, we hypothesized that the structural conversion of the RNA 3′ terminus by Cvfa prevents its degradation by PNPase. To test this hypothesis, we chemically synthesized 3′-hydroxylated RNA (3′-OH RNA), 2′,3′-cyclic RNA, and 3′-phosphorylated RNA using the phosphoramidite method (Fig. 4, A–C), and examined whether the purified PNPase degrades these RNAs. In the case of 3′-OH RNA and 2′,3′-cyclic RNA, the RNA bands disappeared with increasing amounts of PNPase (Fig. 4D). In contrast, the 3′-phosphorylated RNA band did not disappear, even at the highest concentration of PNPase (Fig. 4D). Furthermore, we determined the $K_m$ and $V_{max}$ values of PNPase against these RNA substrates by measuring the dose-response curve of the substrate RNA concentration and RNA degradation activity (Fig. 4, E–G). $V_{max}$ values of PNPase against 3′-OH RNA, 2′,3′-cyclic RNA, and 3′-phosphorylated RNA were 35, 6, and 1 μmol/min/mg protein, respectively (Table 5). In addition, the $K_m$ values against each RNA substrate were 22, 42, and 156 μM, respectively (Table 5). These results suggest that 3′-phosphorylated RNA is resistant to degradation by PNPase.

| Table 5: Kinetic parameters of PNPase activity on different kinds of substrate RNAs |
|-------------------------------------|----------|----------|
| Substrate                          | $V_{max}$ | $K_m$    |
| 3′-OH RNA                          | 35        | 22       |
| 3′-Cyclic RNA                      | 6         | 42       |
| 3′-Phosphorylated RNA              | 1         | 156      |

**FIGURE 5. Expression of PNPase and Cvfa in different culture conditions.** A. S. aureus parent strain (NCTC8325-4) was cultured to $A_{600} = 0.2, 1, or 6$ (15 h) at 37 °C. Cells were collected and subjected to Western blotting with anti-Cvfa IgY or anti-PNPase IgG. Each lane contains 1 μg of cell extract protein. B. S. aureus parent strain (NCTC8325-4) was cultured to $A_{600} = 0.8$ at 37 °C and transferred to 16 °C for 3 h or maintained at 37 °C for 3 h. Cell extracts were subjected to Western blotting with anti-Cvfa IgY or anti-PNPase IgG. Each lane contains 1 μg of cell extract protein. C. S. aureus parent strain (NCTC8325-4), the pnpA-deleted mutant (M117NC), and the cvfA-deleted mutant (CKP1129) were cultured to $A_{600} = 1$ or 6 (15 h) at 37 °C. Cvfa and PNPase were detected by Western blotting. Each lane contains 0.2 μg of protein. D. Eight strains of hospital-associated MRSA (CR1–8 strains) and two strains of community-acquired MRSA (FRP3757 and MW2) were cultured overnight, and the cell extracts were used for Western blotting with anti-Cvfa and anti-PNPase antibodies. Each lane contains 1 μg of protein.
Effect of Growth Phase and Cold Stress on the Expression of CvfA and PNPase—If CvfA and PNPase competitively regulate S. aureus gene expression, the expression ratio of CvfA and PNPase might change under different culture conditions. S. aureus exotoxin expression is stimulated in the stationary phase by the agr quorum-sensing system, whereas it is inhibited in the exponential phase (34). In E. coli, the expression of PNPase is activated at cold temperatures (35, 36). We examined the effects of the growth phase and cold stress on the expression ratio of CvfA and PNPase. Both CvfA and PNPase were constantly expressed from the exponential phase ($A_{600} = 0.2–1$) to the stationary phase ($A_{600} = 6$), and the ratio of CvfA and PNPase did not change (Fig. 5A). In contrast, the amount of PNPase was decreased at 16 °C compared with that at 37 °C, whereas the amount of CvfA was increased at 16 °C compared with that at 37 °C (Fig. 5B). These results suggest that the ratio of CvfA and PNPase changes and affects gene expression under certain conditions.

Because the reciprocal expression of CvfA and PNPase was observed in the cold stress condition, we examined whether CvfA and PNPase are expressed in clinical isolates of S. aureus. S. aureus is a problematic pathogen due to its antibiotic-resistant capacity. We examined 10 clinical isolates of methicillin-resistant S. aureus (MRSA). All tested strains expressed both CvfA and PNPase (Fig. 5D). This finding suggests that the regulation by CvfA and PNPase is not specific to a laboratory strain but is conserved in most S. aureus strains.

### DISCUSSION

The findings of this study indicated that the decreased hemolysin production and agr expression in the cvfA-deleted mutant was suppressed by disruption of pnpA-encoding PNPase with 3'- to 5'-exonuclease activity. The increased hemolysin production in the cvfA/pnpA mutant was complemented by the expression of wild-type PNPase, whereas it was not complemented by the expression of mutated PNPases without RNA degradation activity or without an RNA binding domain (Table 6). Therefore, both RNA degradation activity and the RNA binding domain of PNPase are required for the genetic interaction between cvfA and pnpA. Because the mutated PNPase without an RNA binding domain (PNPase ΔRBD) retained the RNA degradation activity in vitro, PNPase ΔRBD may be defective in capturing and degrading specific RNA substrates in vivo. Furthermore, we demonstrated that 2',3'·cyclic RNA, a substrate of CvfA, is sensitive to PNPase-mediated degradation, whereas 3'-phosphorylated RNA, a product of CvfA, is resistant to PNPase-mediated degradation (Fig. 4). These results suggest that an RNA essential for expression of the agr and hemolysin genes is modified to 3'-phosphorylated RNA by CvfA and escapes degradation by PNPase (Fig. 6). If the RNA is not modified by CvfA, the RNA will be degraded by PNPase. This model can explain why the cvfA-

### TABLE 6

**Characteristics of the mutated PNPases**

Complementation + indicates that PNPase expression decreased the hemolysin production of the cvfA/pnpA double mutant. Phosphorolysis and Polymerization refer to the biochemical activities of PNPase in vitro.

| Phosphorolysis | Polymerization | Complementation |
|----------------|----------------|-----------------|
| Wild-type PNPase | + | + | + |
| D946G | + | + | + |
| R402A/R403A | – | – | – |
| H407D | – | – | – |
| R413D | + | + | + |
| D496G | – | – | – |
| ΔRBD | + | + | – |

![FIGURE 6](https://example.com/image.png)

**FIGURE 6. S. aureus hemolysin production via control of RNA stability by CvfA and PNPase.** A specific RNA (3'-OH RNA) that is required for hemolysin production is cleaved by endonuclease activity of CvfA or other endonucleases and results in the production of 2',3'-cyclic RNA. Next, the 2',3'-cyclic RNA is converted to 3'-phosphorylated RNA by CvfA. 3'-OH RNA and 2',3'-cyclic RNA are degraded by PNPase, whereas 3'-phosphorylated RNA is resistant to PNPase degradation.
deleted mutant exhibits decreased hemolysin production as well as why the cvfA/pnpA-disrupted mutant restores hemolysin production.

Based on microarray and quantitative RT-PCR analysis, we found that the decreased expression of the sae locus in the cvfA-deleted mutant was suppressed by the disruption of the pnpA gene. Based on the report that the cvfA gene affects the processing of the saePQRS transcript (27), saePQRS mRNA might be a target of CvFA and PNPAse. In addition, because expression of the sae locus is positively regulated by the agr locus (34) and cvfA and pnpA regulate the expression of the agr locus in an opposing manner, the effect of cvfA and pnpA on sae expression might be due in part to the altered expression of the agr locus. It is also possible that CvFA and PNPAse directly target mRNAs encoding hemolysins and other virulence factors, whose expression is regulated by sae and agr. We also found that the decreased expression of the adhE gene encoding alcohol dehydrogenase, which is involved in acetate metabolism, was suppressed by the disruption of the pnpA gene. The expression of adhE was not affected by either agr (37) or saeRS (38). Thus, CvFA and PNPAse target RNA genes other than agr and saeRS to control the expression of adhE, and those genes remain to be identified. As a whole, CvFA and PNPAse regulate energy metabolism and virulence in S. aureus. We further demonstrated that the decreased killing abilities of the cvfA-deleted mutant against silkworms and mice were not attenuated by the disruption of pnpA (Table 3). The finding that agr expression, which is required for virulence in both silkworms (26) and mice (39), was restored in the cvfA/pnpA double-disrupted mutant (Fig. 1) suggests the presence of other factors that were not restored in the double mutant. These findings suggest that the target RNAs of CvFA are not totally same as those of PNPAse. Further studies are needed to reveal the characteristics of the target RNAs of CvFA and PNPAse and the mechanism of target recognition by each enzyme.

B. subtilis RNase Y, a homolog of CvFA, has endonuclease activity against mRNA (2, 5, 6). At present, it is unclear whether S. aureus CvFA has endonuclease activity. 2',3'-Cyclic RNA is known to be produced by either endonucleolytic cleavage (40) or by RNA terminal cyclase (41). Bacterial tRNA ligase cleaves immature tRNA to produce 2',3'-cyclic tRNA and further cleaves it to produce 2'-phosphorylated tRNA (42). Based on these reports, CvFA may be a phosphodiesterase against 2',3'-cyclic RNA that is produced by RNA cleavage by CvFA or some other endonuclease or by an RNA terminal cyclase (Fig. 6).

RNA stability can be controlled by modifying the RNA structure by the addition of a 3'-poly(A) tail (43, 44) and the formation of hairpin loop structures (45). To our knowledge, this study is the first to suggest that modification of the 3'-terminal nucleotide structure of RNA controls RNA stability and regulates bacterial virulence. Controlling RNA stability allows a faster response of gene expression to environmental changes than control of RNA transcription. S. aureus hemolysins function in various infectious stages, including lysis of host cells, escape from cellular immunity, and biofilm formation (46–49). The regulation of RNA stability by CvFA and PNPAse might be important for bacteria to quickly regulate the expression of hemolysin genes according to host environmental changes.

Control of RNA stability by modification of the 3'-terminal nucleotide structure of RNA requires little energy consumption and has little effect on the whole RNA secondary structure. Future studies are needed to develop methods to determine the 3'-terminal nucleotide structure of endogenous mRNA and to elucidate the biological significance of the control of RNA stability by altering the 3'-terminal nucleotide structure.

REFERENCES
1. Lehnik-Habrink, M., Lewis, R. J., Mader, U., and Stulke, J. (2012) RNA degradation in Bacillus subtilis: an interplay of essential endo- and exoribonucleases. Mol. Microbiol. 84, 1005–1017
2. Shahbabian, K., Jamall, A., Zieg, L., and Putzer, H. (2009) RNase Y, a novel endoribonuclease, initiates riboswitch turnover in Bacillus subtilis. EMBO J. 28, 3523–3533
3. Andrade, J. M., Pobre, V., Silva, J. J., Domingues, S., and Arraiano, C. M. (2009) The role of 3'-5'-exoribonucleases in RNA degradation. Prog. Mol. Biol. Transl. Sci. 85, 187–229
4. Hunt, A., Rawlins, J. P., Thomaides, H. B., and Errington, J. (2006) Functional analysis of 11 putative essential genes in Bacillus subtilis. Microbiology 152, 2895–2907
5. Commichau, F. M., Rothe, F. M., Herzberg, C., Wagner, E., Hellwig, D., Lehnik-Habrink, M., Caldelari, I., Parmentier, D., Lioliou, E., Romby, P., and Fechner, C. (2011) Role of nucleotide structure at 3' terminus of mRNA to produce 2'-phosphorylated tRNA (42). Based on these reports, CvFA may be a phosphodiesterase against 2',3'-cyclic RNA that is produced by RNA cleavage by CvFA or some other endonuclease or by an RNA terminal cyclase (Fig. 6).

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Role of Nucleotide Structure at 3' Terminus of RNA

human RNA 3'-terminal phosphate cyclase is a member of a new family of proteins conserved in Eucarya, Bacteria and Archaea. Embo J. 16, 2955–2967.

Sidrauski, C., Cox, J. S., and Walter, P. (1996) tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. Cell 87, 405–413.

Xu, F., and Cohen, S. N. (1995) RNA degradation in Escherichia coli regulated by 3’ adenylation and 5’ phosphorylation. Nature 374, 180–183.

Blum, E., Carposuis, A. J., and Higgins, C. F. (1999) Polyadenylation promotes degradation of 3’-structured RNA by the Escherichia coli mRNA degradosome in vitro. J. Biol. Chem. 274, 4009–4016.

Py, B., Higgins, C. F., Krisch, H. M., and Carposuis, A. J. (1996) A DEAD-box RNA helicase in the Escherichia coli RNA degradosome. Nature 381, 169–172.

Jarry, T. M., Memmi, G., and Cheung, A. L. (2008) The expression of α-haemolysin is required for Staphylococcus aureus phagosomal escape after internalization in CFT-1 cells. Cell. Microbiol. 10, 1801–1814.

Caiazza, N. C., and O’Toole, G. A. (2003) Alpha-toxin is required for biofilm formation by Staphylococcus aureus. J. Bacteriol. 185, 3214–3217.

Bubek Wardenburg, J., Patel, R. J., and Schneewind, O. (2007) Surface proteins and exotoxins are required for the pathogenesis of Staphylococcus aureus pneumonia. Infect. Immun. 75, 1040–1044.

Bubek Wardenburg, J., Bae, T., Otto, M., Deleo, F. R., and Schneewind, O. (2007) Pore forming pores α-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. Nat. Med. 13, 1405–1406.

Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J., and Schleier, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus. J. Bacteriol. 170, 4363–4372.

Kaito, C., Saito, Y., Ikou, M., Omae, Y., Mao, H., Nagano, G., Fujiyuki, T., Numata, S., Han, X., Obata, K., Hashgawa, S., Yamaguchi, H., Inokuchi, K., Ito, T., Hiramatsu, K., and Sekimizu, K. (2013) Mobile genetic element SCCmec-encoded psm-mec RNA suppresses translation of agrA and attenuates MRSA RNA degradation. PLoS Pathog. 9, e1003269.

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mondongin, E. F., Sensabaugh, G. F., and Perdue-Remington, F. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant Staphylococcus aureus. Lancet 367, 731–739.

Naimi, T. S., LeDell, K. H., Boxrud, D. J., Groom, A. V., Steward, C. D., Johnson, S. K., Besser, J. M., O’Boyle, C., Danila, R. N., Cheek, J. E., Osterholm, M. T., Moore, K. A., and Smith, K. E. (2001) Epidemiology and clonal diversity of community-acquired methicillin-resistant Staphylococcus aureus in Minnesota, 1996–1998. Clin. Infect. Dis. 33, 990–996.

Moriya, S., Tsujikawa, E., Hassan, A. K., Asai, K., Kodama, T., and Ogasawa, N. (1998) A Bacillus subtilis gene-encoding protein homologous to eukaryotic SMG-5 protein motor is necessary for chromosomes partitioning. Mol. Microbiol. 29, 179–187.

Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998) A vector for systematic gene inactivation in Bacillus subtilis. Microbiology 144, 3097–3104.

Matsuo, M., Kurokawa, K., Nishida, S., Li, Y., Takimura, H., Kaito, C., Fukuhara, N., Maki, H., Miura, K., Murakami, K., and Sekimizu, K. (2003) Isolation and mutation site determination of the temperature-sensitive murB mutants of Staphylococcus aureus. FEMS Microbiol. Lett. 222, 107–113.

Matsumoto, Y., Kaito, C., Morishita, D., Kurokawa, K., and Sekimizu, K. (2007) Regulation of exoprotein gene expression by the Staphylococcus aureus cvfB gene. Infect. Immun. 75, 1964–1972.

Bermúdez-Cruz, R. M., Fernández-Ramírez, F., Ramírez, F., Kameyama, T., Fukuhara, N., Maki, H., Miura, K., Murakami, K., and Sekimizu, K. (2003) Isolation and mutation site determination of the temperature-sensitive murB mutants of Staphylococcus aureus. FEMS Microbiol. Lett. 222, 107–113.

Matsuo, M., Kurokawa, K., Nishida, S., Li, Y., Takimura, H., Kaito, C., Fukuhara, N., Maki, H., Miura, K., Murakami, K., and Sekimizu, K. (2003) Isolation and mutation site determination of the temperature-sensitive murB mutants of Staphylococcus aureus. FEMS Microbiol. Lett. 222, 107–113.