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Cyclic di-AMP mediates biofilm formation

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Summary

Cyclic di-AMP (c-di-AMP) is an emerging second messenger in bacteria. It has been shown to play important roles in bacterial fitness and virulence. However, transduction of c-di-AMP signaling in bacteria and the role of c-di-AMP in biofilm formation is not well understood. The level of c-di-AMP is modulated by activity of di-adenylyl cyclase that produces c-di-AMP and phosphodiesterase (PDE) that degrades c-di-AMP. In this study, we determined that increased c-di-AMP levels by deletion of the *pdeA* gene coding for a PDE promoted biofilm formation in *Streptococcus mutans*. Deletion of *pdeA* up-regulated expression of *gtfB*, the gene coding for a major glucan producing enzyme. Inactivation of *gtfB* blocked the increased biofilm by the *pdeA* mutant. Two c-di-AMP binding proteins including CabPA (SMU_1562) and CabPB (SMU_1708) were identified. Interestingly, only CabPA deficiency inhibited both the increased biofilm formation and the up-regulated expression of GtfB observed in the *pdeA* mutant. In addition, CabPA but not CabPB interacted with VicR, a known transcriptional factor that regulates expression of *gtfB*, suggesting that a signaling link between CabPA and GtfB through VicR. Increased biofilm by the *pdeA* deficiency also enhanced bacterial colonization of *Drosophila in vivo*. Taken together, our studies reveal a new role of c-di-AMP in mediating biofilm formation through a CabPA/VicR/GtfB signaling network in *S. mutans*.

Keywords

Streptococcus mutans; biofilms; c-di-AMP

Introduction

Signaling molecules are crucial for bacterial physiology and pathogenesis (Kalia *et al*., 2013, Antunes *et al*., 2010, Minvielle *et al*., 2013). The best known and well characterized bacterial signaling molecules are autoinducers, which mediate the quorum sensing systems, such as competence stimulating peptide in Gram-positive bacteria and homoserine lactones in Gram-negative bacteria (Senadheera & Cvitkovitch, 2008, Cvitkovitch, 2001, Kim *et al*.,

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2013, Antunes *et al.*, 2010). Nucleotide signaling molecules such as cyclic AMP (cAMP) and (p)ppGpp have been demonstrated in mediating carbon metabolism and stringent response (Gomelsky, 2011, Kalia *et al.*, 2013). Recently, cyclic di-GMP (c-di-GMP), a cyclic dinucleotide, has been recognized as a key signaling molecule involved in regulating bacterial biofilm formation and virulence (Hengge, 2009, Gomelsky, 2011, Römling *et al.*, 2013, Nunes-Alves, 2014).

Cyclic di-AMP (c-di-AMP) is a newly discovered cyclic dinucleotide (Corrigan & Grundling, 2013). c-di-AMP is an essential signaling molecule in a wide variety of bacteria (Zhang *et al.*, 2012, Kamegaya *et al.*, 2011, Woodward *et al.*, 2010, Witte *et al.*, 2008, Corrigan *et al.*, 2011, Bai *et al.*, 2013). It is synthesized from two molecules of ATP by di-adenylyl cyclase (DAC) enzymes and degraded to pApA or AMP by distinct c-di-AMP phosphodiesterase enzymes (PDE) (Bai *et al.*, 2013, Rao *et al.*, 2010). DAC enzymes have been identified in a large number of bacteria (Woodward *et al.*, 2010, Kamegaya *et al.*, 2011, Bejerano-Sagie *et al.*, 2006, Barker *et al.*, 2013, Corrigan *et al.*, 2011). These enzymes contain a conserved DisA_N domain (recently renamed as DAC) (Kamegaya *et al.*, 2011, Woodward *et al.*, 2010, Bejerano-Sagie *et al.*, 2006, Barker *et al.*, 2013, Corrigan *et al.*, 2011). Most bacteria, including *Streptococcus mutans*, only possess one DAC domain containing protein (Mehne *et al.*, 2013). The first c-di-AMP degrading phosphodiesterase enzyme named GdpP was discovered in *Bacillus subtilis* (Rao *et al.*, 2010). GdpP contains a PAS sensory domain, a highly variable GGDEF domain, a DHH domain, and a DHH-associated DHHA1 domain. The C-terminal DHH–DHHA1 region is essential for PDE activity (Corrigan *et al.*, 2011). A new type of c-di-AMP phosphodiesterase, PgpH, which contains an HD domain, was recently found in *Listeria monocytogenes* (Huynh *et al.*, 2015), but no PgpH homolog was found in *S. mutans*. *S. mutans* has only one protein (PdeA) that contains GGDEF and DHH-DHHA domains. c-di-AMP plays important roles in regulating diverse cellular pathways. It modulates fatty acid synthesis and transport in *Mycobacterium smegmatis* (Zhang *et al.*, 2013), and alters bacterial cell size and regulates cell envelope stress and biofilm formation in *Staphylococcus aureus* (Corrigan *et al.*, 2011). Moreover, changes in c-di-AMP levels influence potassium uptake, peptidoglycan homeostasis, acid tolerance, antibiotic resistance, and bacterial virulence (Corrigan *et al.*, 2011, Rao *et al.*, 2010, Bai *et al.*, 2013, Luo & Helmann, 2012). Increased c-di-AMP levels affect growth and promote biofilm formation in *Streptococcus suis* (Du *et al.*, 2014). However, factors connecting c-di-AMP and biofilm formation are unknown.

S. mutans is one of the primary etiologic agents of dental caries. Formation of biofilm is a key virulence feature of this pathogen. *S. mutans* synthesizes extracellular polysaccharides (EPS) to promote biofilm formation using distinct glucosyltransferases (GTFs) (Koo *et al.*, 2010). The EPS, including both water-insoluble and water-soluble glucans, mediates the initial adherence of *S. mutans* and other oral bacteria to the tooth surface and facilitates the formation of mature dental plaque (Rölla, 1989, Koo *et al.*, 2009). GtfB, a major enzyme responsible for production of water-insoluble glucans, is critical for biofilm formation and virulence of *S. mutans* (Yamashita et al., 1993). GtfB deficiency in *S. mutans* inhibits biofilm formation and reduces bacterial virulence (Koo *et al.*, 2002, Koo *et al.*, 2010). However, it is unknown what signal modulates expression of *gtfB*. Understanding the underlying regulatory mechanisms of biofilm formation by the new bacterial second

messenger will help to uncover new targets that are amenable to development of therapeutics to prevent and treat dental caries.

In this study, we showed that c-di-AMP mediated *S. mutans* biofilm formation. Increased c-di-AMP levels concurrently promoted biofilm formation via up-regulating the expression of *gtfB*. The enhanced biofilm formation and increased GtfB were simultaneously blunted by deletion of either a c-di-AMP binding protein (CabPA) or GtfB. Further we found that CabPA interacted with VicR, a response regulator of the VicRK two-component system, that modulates biofilm formation (Smith & Spatafora, 2012, Senadheera *et al.*, 2012). Together, our studies demonstrate that c-di-AMP controls *S. mutans* biofilm formation by regulation of *gtfB* expression through the binding of CabPA to VicR. This study defines a new signaling network that links c-di-AMP signaling and biofilm formation.

Results

PdeA modulates cellular c-di-AMP levels in *S. mutans*

c-di-AMP PDEs have been identified in diverse bacteria. *S. mutans* PdeA (SMU_2140c) is a homolog of GdpP that possesses a DHH-DHHA1 domain (FIG. S1). To determine whether PdeA possesses c-di-AMP PDE activity, we generated an in-frame deletion of *pdeA* and measured the amount of intracellular c-di-AMP produced by the mutant. The amount of intracellular c-di-AMP was increased approximately 6-fold in the *pdeA* deficient mutant when compared to that in the wild type. Complementation of the mutant with the full-length *pdeA* gene partially restored the intracellular c-di-AMP levels (FIG. 1A). These data suggest that PdeA functions as a c-di-AMP PDE in *S. mutans*.

The PdeA deficiency promotes biofilm formation of *S. mutans*

To investigate how the PdeA deficiency affects the ability of *S. mutans* to form biofilms, we performed biofilm assays first using a crystal violet staining method. The *pdeA* mutant produced significantly more biomass than the wild type bacteria, although the *pdeA* mutant exhibited a decreased growth (FIG. S2). The complemented strain partially restored the wild type phenotype (FIG. 1B). Similar phenotypes were observed when biofilm formation, and production of c-di-AMP of the *pdeA* mutant were monitored at different time points (8–24 hours) (FIG. S3).

The effect of the PdeA deficiency on biofilm structure of *S. mutans* was also evident as assessed by glucan labeling and confocal laser scanning microscopy (CLSM). The wild type *S. mutans* formed typical biofilm clusters, while the *pdeA* mutant showed slightly altered distribution of cells and matrix, and markedly enhanced production of EPS when probed by the labeled dextran (FIG. 2A) (Xiao *et al.*, 2012). Quantification of biofilm images (FIG. 2A) by the COMSTAT program and vertical sectioned biofilm images (FIG. 2B) also revealed that the *pdeA* mutant formed much thicker biofilm, produced more EPS in comparison with the wild type bacteria (FIG. 2C). Interestingly the *pdeA* mutant retained the bacterial level while produced more EPS (FIG. 2C, the right panel). Complementation of the *pdeA* mutant partially restored these phenotypes, demonstrating the role of PdeA in regulating biofilm formation. We also examined a recombinant strain that overexpressed

DAC (the enzyme produces c-di-AMP) and found overexpressed DAC increased biofilm formation when compared to wild type (FIG. 3D). The PdeA deficiency and overexpression of DAC both increased c-di-AMP levels (FIG. 1A&S5) and promoted sucrose-dependent biofilm formation. Taken together, these data imply that c-di-AMP positively regulates biofilm formation.

c-di-AMP binding protein CabPA is crucial for the biofilm formation

To investigate how c-di-AMP regulates biofilm formation in *S. mutans*, we performed an affinity purification to identify proteins that bind c-di-AMP by using 2'-AHC-c-diAMP-agarose resin (Biolog Life Sciences Institute) (Bai *et al.*, 2014). Two proteins were evident in the eluted fraction and identified by LC/MS/MS as CabPA with a molecular mass of ~25 kDa, and CabPB with a molecular mass of ~55 kDa (FIG. 3A). Both CabPA and CabPB contain TrkA_C and TrkA_N domains (FIG. S1), and the TrkA domains were found bound peripherally to the inner side of cytoplasmic membrane of the constitutive potassium-uptake systems (Schlosser *et al.*, 1993), the TrkA_C domain containing proteins have been identified as c-di-AMP receptors (Corrigan *et al.*, 2013). To further verify that both CabPA and CabPB are c-di-AMP binding proteins, we performed an *in vitro* pull-down assay using biotinylated c-di-AMP coupled beads. Both CabPA and CabPB were readily pulled down by c-di-AMP-coupled beads (FIG. 3B). Additionally, a differential radial capillary action of ligand assay (DRaCALA) was performed to verify the bindings. CabPA had lower affinity for c-di-AMP (K_d = 7.8±0.3 μM), when compared to CabPB (K_d= 1.2±0.2 μM), which is consistent with the observation that less CabPA was co-purified than CabPB by the pull-down assays (FIG. 3B&3C).

To determine whether CabPA and CabPB are responsible for the increased biofilm formation seen in the *pdeA* mutant, we mutated *cabPA* and *cabPB*, respectively, in the *pdeA* deficient background, and then examined their biofilms. *cabPA* deficiency in the *pdeA* mutant background inhibited the enhanced biofilm formation, and complementation of the *pdeA-cabPA* double mutant with *cabPA* restored the enhanced biofilm formation. In contrast, inactivation of *cabPB* in the *pdeA* mutant background did not alter the increased biofilm formation (FIG. 3D&S4). To determine whether these effects are due to changes in the c-di-AMP levels, we measured the c-di-AMP levels of all mutants and the DAC overexpressing strain, and found that enhanced biofilm formation is linked to increased c-di-AMP levels (FIG. S5). To determine regulation of CabPA by c-di-AMP produced from the *pdeA* mutant in the biofilm formation, we performed epistasis experiments. We first attempted to restore biofilm defects of *cabPA*, *pdeA*, and *cabPA-pdeA* by provision of *cabPA* and *pdeA* on a plasmid (FIG. 3D). Overexpression of *cabPA* restored the biofilm phenotype of the *cabPA* mutant, and significantly increased biofilm formation of the *pdeA* mutant, suggesting increased c-di-AMP coupled with overexpressed CabPA promoted biofilm formation. On the other hand, the *pdeA* gene restored the biofilm phenotype of the *pdeA* mutant, but failed to alter biofilm phenotype of the *cabPA* mutant (Fig. 3D). Together, these data suggest that CabPA is a key c-di-AMP receptor that is required for the c-di-AMP-mediated biofilm formation.

c-di-AMP regulates GtfB-dependent biofilm formation

To investigate how c-di-AMP mediates biofilm formation in *S. mutans*, we attempted to isolate genes involved in c-di-AMP-mediated biofilm by a transposon mutagenesis scheme. A transposon library on the *pdeA* mutant background was generated using a *Himar 1* Mariner mini-transposon system (van Opijnen & Camilli, 2010), and validated as described (Klein *et al.*, 2012). Over 2000 individual transposon mutants were screened to isolate the mutants that would have reduced c-di-AMP-mediated biofilm formation. Two transposon mutants that exhibited lower biofilm biomass were identified and confirmed. Sequencing analysis of the transposon mutants revealed the transposon element inserted into *gtfB* (FIG. 4) and *smu_30* (data not shown), respectively. *gtfB* encodes a glucosyltransferase that is critical to biofilm formation in *S. mutans* while *smu_30* codes for a hypothetical protein with no known function in the biofilm formation. Complementation of the *pdeA-gtfB* double mutant with *gtfB* restored the enhanced biofilm formation (FIG. 4). Overexpression of *gtfB* in the *cabPA* mutant significantly increased biofilm formation, while overexpression of either *pdeA* or *cabPA* in the *gtfB* mutant didn't restore biofilm formation, suggesting that GtfB is a downstream factor.

c-di-AMP regulates Gtf-dependent EPS production

Since GtfB deficiency blunted increased biofilm formation in the *pdeA* mutant (FIG. 4), EPS production was enhanced in the *pdeA* mutant (FIG. 2A & B) and EPS is primarily synthesized by three glucosyltransferases GtfB, GtfC and GtfD, we examined how the PdeA deficiency affected expression of *gtfB, gtfC* and *gtfD* by real-time RT-PCR. Interestingly, the expression of *gtfB* in the *pdeA* mutant was up-regulated by 12-fold compared to the wild type; however there was no significant difference in expression of *gtfC* and *gtfD* (FIG. 5A). Furthermore, inactivation of *cabPA* but not *cabPB* abolished the increased expression of *gtfB* by the *pdeA* mutant (FIG. 5A). We subsequently examined the effect on the GtfB protein level using Western blot analysis. Consistently, the amount of GtfB was significantly increased in the *pdeA* mutant and restored to the wild type level when *cabPA* but not *cabPB* was deleted in the *pdeA* mutant (FIG. 5B&5C). To investigate whether c-di-AMP affects secreted GtfB, we examined GtfB in culture supernatants and found that secreted GtfB was altered similarly (FIG. S6). These findings are consistent with the changes in the biofilm formation by various mutants (FIG. 3D&S4). Other biofilm associated or glucan binding related surface proteins such as antigen I/II, GbpA and GbpD were not significantly affected (FIG. 5B), suggesting selective impact of c-di-AMP on GtfB through CabPA. Together, these data indicate that c-di-AMP, the binding receptor CabPA, and the production of GtfB are tightly coordinated with the biofilm formation in *S. mutans*, which constitutes a major c-di-AMP-mediated biofilm pathway. Interestingly, we found the *pdeA-cabPA* double mutant had more GtfB than the *cabPA* single mutant and produced more biofilm biomass (FIG. 5B, 5C&3D), indicating that CabPA-independent biofilm formation pathways also exist in response to elevated c-di-AMP (FIG. 8).

Binding of CabPA to VicR, a known response regulator that mediates expression of *gtfB*

The above studies suggest that CabPA may regulate expression of *gtfB*. To determine how CabPA regulates GtfB. We used TAP (**T**andem **A**ffinity **P**urification) method to identify

proteins that associate with CabPA *in vivo* in *S. mutans*. We found a ~25kDa protein was specifically purified with CabPA (FIG. 6A), and this band is evident by protein staining. It was determined to be VicR by LC/MS/MS. VicR is a response regulator of a known *S. mutans* two-component system. To confirm this we probed the purified proteins with VicR antiserum, the western blot demonstrated that the protein associated with CabPA is VicR. When other biofilm relevant proteins GbpA and D (Glucan-binding proteins) and BrpA (Biofilm regulatory protein, a putative transcriptional factor and important for biofilm formation), were probed by their antibodies, GbpC non-selectively bound to TAP purified proteins independent of CabPA, while BrpA was not detectable (FIG. S7), demonstrating the specific association of VicR with CabPA. To determine whether CabPA directly interacts with VicR, we performed a pull-down assay using recombinant CabPA, CabPB and VicR, and confirmed that VicR directly interacted with CabPA not CabPB *in vitro* (FIG. 6B). Therefore, both *in vivo* and *in vitro* data demonstrated that CabPA binds VicR. Overall, our results suggest that c-di-AMP mediates GtfB expression and biofilm formation through the interaction between CabPA and VicR.

PdeA deficiency promotes colonization of *Drosophila* by *S. mtuans*

To determine if c-di-AMP impacts *S. mutans* colonization *in vivo*, we employed a widely used *Drosophila* colonization model (Mulcahy *et al.*, 2011, Chugani *et al.*, 2001). Flies were infected with mid-log phase bacterial cells of wild type *S. mutans*, *gtfB* and *pdeA* mutants. After 2 days of infection, there was no difference in colonization between wild type and the *pdeA* mutant, while the *gtfB* mutant, a known biofilm defective strain, showed significant reduction in colonization. However after 4 days and 6 days of infections, the difference emerged. Wild type *S. mutans* colonized in *Drosophila* at a considerable level of 3.8×10^4 per fly on day 6 post-infection while GtfB deficiency reduced colonization to 4.0×10^3 per fly, about a 10-fold decrease compared to that of the wild type. However, PdeA deficient mutant exhibited significantly increased bacterial colonization to 2.5×10^5 per fly, approximately a six-fold increase over the wild type bacteria (FIG. 7A). To visually show the colonization of Drosophila by *S. mutans*, we fed flies with green fluorescent protein (*gfp*)-tagged strains and examined effects of various mutations on bacterial colonization as described (Dionne *et al.*, 2003). On day 6 post-infection, significantly higher fluorescence intensity was observed in guts of flies fed with the PdeA/*gfp* strain when compared to those fed with WT/*gfp*. Deletion of *gtfB* from the PdeA/*gfp* strain rendered the infected flies exhibit the minimal fluorescence similar to the flies infected with GtfB/gtf (FIG. 7B). These data again demonstrated that increased c-di-AMP promotes colonization of *Drosophila* by *S. mutans in vivo*, and the enhanced colonization by increased c-di-AMP was attenuated by the GtfB deficiency.

Discussion

Biofilm formation is an important virulence determinant of many bacterial pathogens including *S. mutans*. As an emerging bacterial second messenger, c-di-AMP plays an important role in bacterial fitness and virulence (Woodward *et al.*, 2010, Corrigan *et al.*, 2011, Oppenheimer-Shaanan *et al.*, 2011, Bai *et al.*, 2014, Ye *et al.*, 2014, Skrnjug *et al.*, 2014, Yang *et al.*, 2014). However, its role in bacterial biofilm formation is not well defined.

In this study, we determined that c-di-AMP regulates biofilm formation of the oral pathogen *S. mutans*. Our studies revealed a new signaling network that integrates c-di-AMP signaling and biofilm formation of *S. mutans* (FIG. 8).

c-di-AMP signaling is essential for bacterial viability in many Gram-positive bacteria (Corrigan *et al.*, 2011, Bai *et al.*, 2012, Ye *et al.*, 2014, Bai *et al.*, 2013, Luo & Helmann, 2012). It participates in an array of activities, including cell wall biosynthesis, potassium homeostasis, DNA damage response, and bacterial host interactions. For instance, the increase in cross-linked peptidoglycan subunits in *S. aureus gdpP* mutant strengthens the peptidoglycan integrity and renders the bacteria more resistant to antibiotics. c-di-AMP has been implicated in the regulation of biofilm formation of *S. aureus* (Corrigan *et al.*, 2011); however, whether it is directly involved in biofilm formation is unknown. Our studies reveal that regulation of GtfB, a major biofilm matrix building protein, by c-di-AMP and its receptor CabPA via binding VicR is the key step. Glucan binding proteins A, C and D have been implicated in the biofilm formation (Lynch *et al.*, 2013); however, there was no apparent effect of increased c-di-AMP on these three proteins and other closely related glucosyltransferase genes *gtfC* and *gtfD* in *S. mutans*, suggesting the key and selective action through GtfB. Two c-di-AMP receptors from *S. mutans*, CabPA and CabPB, were identified by using c-di-AMP affinity purification scheme. TrkA_C domain is known c-di-AMP binding region (Corrigan *et al.*, 2013). The homologous proteins have been shown to mediate potassium homeostasis in *S. aureus* (Corrigan *et al.*, 2013) and *S. pneumoniae* (Bai *et al.*, 2014). However, whether they are involved in regulating biofilm formation is unknown. Interestingly, although CabPB possesses two TrkA_C domains and exhibited higher affinity for c-di-AMP, inactivation of *cabPB* did not alter the increased biofilm formation in the *pdeA* mutant. In contrast, inactivation of CabPA blunted the increased biofilm in the *pdeA* mutant, again indicating the selective action by CabPA in modulating formation of biofilms. Consistently, inactivation of *cabPA*, not *cabPB*, down-regulated expression of GtfB. These observations provide convincing evidence that CabPA not CabPB regulates biofilm formation of *S. mutans*. Complementation using *cabPA* not *pdeA* restored the biofilm defect of a *cabPA-pdeA* double mutant and overexpression of *cabPA* in the *pdeA* mutant significantly increased biofilm formation (FIG. 3D), further indicating higher concentrations of c-di-AMP can interact with overexpressed CabPA to enhance biofilm formation. Furthermore, our studies indicate the receptor CabPA interacted with VicR, an essential response regulator that has been shown to mediate expression of *gtfB* (Senadheera *et al.*, 2005). These data uncovered a missing link between c-di-AMP signaling and GtfB-mediated biofilm formation.

It is not clear whether potassium homeostasis plays a direct role in biofilm formation. Studies from *B. subtilis* hint a potential association (Lundberg *et al.*, 2013), albeit the underlying mechanism is not known. In addition, up-regulation of a putative potassium uptake system has been associated with sucrose-dependent biofilm formation in *S. mutans* (Klein *et al.*, 2010). Further studies are needed to examine association between potassium homeostasis and the VicRK system on the regulation of biofilm formation.

Intriguingly the *pdeA-cabPA* double mutant produced slightly more GtfB than the *cabPA* single mutant, also formed slightly more biofilms, which may indicate that *cabPA*-independent biofilm pathway exists in response to increased c-di-AMP.

Like c-di-GMP, a well characterized bacterial second messenger that regulates biofilm formation, c-di-AMP promotes biofilm formation by up-regulating production of glucans, a key component of the biofilm matrix. In c-di-GMP mediated biofilm formation, both extracellular matrix components and biofilm associated adhesins are up-regulated (Hengge, 2009). In contrast, c-di-AMP-mediated biofilm enhancement promoted production of glucans, but not other key biofilm associated adhesins such as antigen I/II (Munro *et al.*, 1993), GbpA, and GbpD (Banas & Vickerman, 2003), suggesting that distinct regulation of biofilm formation by c-di-AMP in *S. mutans*. Like c-di-GMP, c-di-AMP has been implicated in modulating bacterial response to a variety of environmental stresses such as antibiotics exposure, acid, and oxidative stress (Romling, 2008, Gomelsky, 2011, Corrigan & Grundling, 2013). Unlike c-di-GMP, c-di-AMP signaling is essential for bacterial viability in many species (Mehne *et al.*, 2013, Dengler *et al.*, 2013, Yamamoto *et al.*, 2012, Bai *et al.*, 2013). Biofilm phenotypes mediated by c-di-AMP may represent a new activity independent of this essential function.

In conclusion, we determined a c-di-AMP-mediated biofilm signaling network, in which the c-di-AMP receptor CabPA controls c-di-AMP-mediated expression of a key biofilm matrix regulator, GtfB, and biofilm formation via the binding to VicR. Alternative biofilm pathways may exist to complement this major pathway. Future studies are warranted to dissect other biofilm pathways and receptors involved and c-di-AMP-mediated stress responses, which would provide a more complete picture for c-di-AMP-mediated biological activities in *S. mutans*.

Experimental procedures

Bacterial strains and culture conditions

S. mutans UA159, *Escherichia coli* Top 10 and BL-21 were used in this study. *S. mutans* strains were grown statically at 37°C in biofilm medium (BM) (Netuschil *et al.*, 1996, Loo *et al.*, 2000), Todd-Hewitt broth (THB) or on TH agar plates under an aerobic atmosphere with 5% CO_2. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates. Appropriate antibiotics were used to select transformants on media as needed.

Construction and complementation of mutants

Mutant strains were derived from *S. mutans* UA159 using PCR ligation mutagenesis with the insertion of a nonpolar kanamycin resistance cassette to replace targeting genes (Ahn *et al.*, 2006, Xie *et al.*, 2011). Briefly, primers (Listed in Table 2) were used to amplify the regions flanking *pdeA*, *cabPA*, and *cabPB*, corresponding PCR products were ligated to kanamycin or erythromycin resistance cassette by fusion PCR, and the obtained PCR products were transformed into competent *S. mutans*. Transformants were selected on THB agar with kanamycin (1 mg ml^{-1}) or erythromycin (10 μg ml^{-1}). PCR and DNA sequencing methods were used to verify whether a correct mutation was introduced. The complete open

reading frame of *pdeA* was amplified and cloned into the *E. coli*-Streptococcus shuttle vector pVPT (Zhou *et al.*, 2008) to generate the corresponding complementation plasmid pVPT-pdeA. The plasmid was then transformed into the *pdeA* mutant strain to generate a complemented strain.

Detection of c-di-AMP

Bacteria strains were grown in 5 ml THB for 16 h, and the growth was determined at an optical density at 470 nm (OD_{470}). The bacteria were harvested by centrifugation at 8,000 rpm for 3 min. The pellets were suspended in 0.5 ml 50 mM Tris-HCl (pH 8.0), sonicated for 20 s, and followed by heating for 10 min at 95°C. Bacterial debris was removed and the supernatant was collected and used to measure c-di-AMP levels by an enzyme-linked immunosorbent assay (ELISA) as described (Bai *et al.*, 2013). The amount of c-di-AMP was normalized by OD_{470} representing of the bacterial growth.

Biofilm formation assay

S. mutans biofilms were grown in either THB containing 1% sucrose or a chemically defined biofilm medium (BM) containing 1% sucrose (Loo *et al.*, 2000). Overnight cultures were subcultured into fresh THB, grown to an OD_{470} of 0.6 and diluted 1:100 and subsequently aliquoted in a 96 well microtiter plate and grown at 5% CO_2 at 37 °C under static conditions (Li *et al.*, 2013). Biofilm samples were collected after 16 h and stained with crystal violet. Crystal violet staining at OD_{562} was used to monitor biofilm formation as described previously (Wu *et al.*, 2007). Due to the different growth rates of wild type and mutants, the biofilms were normalized by bacterial growth using OD_{562}/OD_{470}. Each assay was carried out with duplicate samples and replicated three times.

Confocal laser scanning microscopy (CLSM) analysis

Bacteria strains were grown in BM with 1μM dextran-conjugated alexa (Molecular Probes, Invitrogen) to label EPS on glass coverslips placed in wells of a sterile 6-well cell culture plate (Corning Costar Corp.) under 5% CO_2 at 37°C for 16 h. The biofilm samples were gently washed with PBS three times to remove unattached cells, dried for 5 min, and then stained with SYTO 9 (Molecular Probes, Invitrogen) (Decker *et al.*, 2014). The stained samples were then examined by CLSM (LSM 710; Zeiss) with a 63× oil immersion objective. Images were obtained from serial optical sections and captured at 488nm. At least three independent experiments were performed by the same person on different days, and image stacks were acquired randomly. Thickness and bio-volume of biofilm were quantified by the program COMSTAT (Heydorn *et al.*, 2000, Kreth *et al.*, 2004).

Generation and screening of transposon mutant library

Genomic DNA was isolated from the *pdeA* mutant using Gentra Puregene Yeast/Bact. Kit (Qiagen). The *smupdeA* transposon library was generated as described by Tim *et al* (van Opijnen & Camilli, 2010). Briefly, 1μg of the mutant genomic DNA was incubated in the presence of purified *HimarC9* transposase and 1μg of plasmid *magellan6* as a donor of the *Himar1* Mariner transposon with spectinomycin resistance. After repair of the resulting transposition products with T4 DNA polymerase and *E. coli* DNA ligase , 1 μg mutagenized

DNA was used for transformation by using natural transformation for *S. mutans* (Liu *et al.*, 2011). The randomness of transposon library was determined by sequencing 10 colonies randomly picked from the library. To screen the library for mutants that have biofilm defects, we performed a crystal violet assay to measure biofilm formation of 2000 individual insertion mutants. Mutants that had significant lower biofilm formation than the *pdeA* mutant were identified, transposon insertion was mapped as described (van Opijnen & Camilli, 2010).

Affinity purification of c-di-AMP binding proteins

Thirty milliliters of an overnight *S. mutans* culture were harvested and suspended in 1 mL 10 mM Tris·HCl (pH 7.5), 50 mM NaCl buffer containing EDTA-free complete protease inhibitor (Roche). Purification of c-di-AMP binding proteins from *S. mutans* cell lysates was carried out by using the previously described method (Bai *et al.*, 2014). For *in vitro* affinity pull-down assay, forty microliters streptavidin dynabeads (Invitrogen) coupled with 2.4 μM biotinylated c-di-AMP (Biolog Life Sciences Institute) were incubated with 5 μM of purified recombinant CabPA or CabPB in 1.5 mL 10% (vol/vol) glycerol, 1 mM $MgCl_2$, 5 mM Tris (pH 7.5), 250 mM NaCl, 0.5 mM DTT, and 4 mM EDTA containing 50 μg mL^{-1} BSA for 30 min at room temperature. Samples were washed four times with the same buffer lacking BSA and suspended in 50 μL protein sample buffer. Samples were boiled for 5 min to release the protein binding to c-di-AMP beads, centrifuged to remove beads, and the supernatant samples were analyzed using SDS-PAGE (Corrigan *et al.*, 2013).

Tandem affinity purification

pVPT-*tap*-CabPA was constructed by PCR amplification of *cabPA* from *S. mutans* UA159 chromosomal DNA using primers CabPA-SalI-F/CabPA-KpnI-R (Table 2), digestion with SalI and KpnI, and ligation into pVPT-*tap*. The tandem affinity purification (TAP) methods provide a tool that allows rapid purification under native conditions of complexes (Puig *et al.*, 2001, Rohila *et al.*, 2004). Tandem affinity purification was performed as described (Puig *et al.*, 2001, Wu & Wu, 2011) with modification. In brief, 500 ml of overnight *S. mutans* cultures were harvested and frozen at −80 °C. The pellets were thawed by adding 30 ml NETN (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5% (v/v) NP-40) and lysed by sonication (10 min, 5 s on, 10 s off). The lysates were then centrifuged (20 min, 10000 rpm, 4°C). The supernatants were transferred into another tubes and centrifuged again. The purification was carried out as described except using NETN buffer.

Mass Spectrometry

The amples were subjected to 10% SDS-PAGE analysis and Comassie blue staining. Potein bands were excised from the gels and digested with trypsin. The digested peptide fragments were analyzed for protein identification by liquid chromatography-tandem mass spectrometry (LC/MS/MS) as described previously (Bu *et al.*, 2008).

Differential Radial Capillary Action of Ligand Assay (DRaCALA)

DRaCALA is a method based on the ability of dry nitrocellulose to separate the free ligand from bound protein–ligand complexes, which allows detection of specific interactions

between nucleotides and their cognate binding proteins (Roelofs *et al.*, 2011, Corrigan *et al.*, 2013). Briefly, [α-^{32}P] ATP (MP Biomedicals) was converted to ^{32}P-labeled c-di-AMP by using recombinant *M. tuberculosis* DisA protein and purified using a thin layer chromatography (TLC) (Bai *et al.*, 2014). The reaction (10 μl) for DRaCALA assay was prepared by mixing 1 μL ^{32}P-labeled c-di-AMP and appropriate concentrations of recombinant CabPA or CabPB in binding buffer (40 mM Tris, 100 mM NaCl, 20 mM $MgCl_2$ [pH 7.5]). Reaction was incubated at room temperature for 10 min, followed by spotting 5 μL onto a nitrocellulose membrane (GE healthcare). After air dry for 10 min, the membrane was exposed on a phosphor screen for 3 h. The radioactivity was detected using a Storm 860 PhosphorImager (Molecular Dynamics). Dissociation constant was analyzed as previous reported (Roelofs *et al.*, 2011).

Expression and purification of CabPA and CabPB

Recombinant CabPA and CabPB constructs were created by amplifying the open reading frames of *cabPA* and *cabPB* from *S. mutans* genomic DNA and ligating into the pET-28(b) vector, each introducing a C-terminal 6×His tag using primers listed in Table 2. The recombinant plasmids were transformed into *E. coli* BL-21(DE3), and induction of protein expression was analyzed by SDS-PAGE. One liter of bacterial culture for each induced recombinant strain was pelleted by centrifugation, resuspended in 30 mL lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl), and lysed by sonication (10 min, 5 s on, 10 s off). Cell debris was pelleted by centrifugation for 60 min followed by passing through a HisTrap column (Ni-affinity column). The proteins were further purified using a 16/60 Superdex 75 gel-filtration column (GE Healthcare) to near homogeneity and were maintained in a solution containing 20 mM Tris–HCl (pH 8.0) and 100 mM NaCl (Zhu *et al.*, 2013).

Transcriptional analysis by qRT-PCR

Total RNA was extracted from the same numbers of harvested bacterial cells from the wild type control and mutants groups. All harvested cells were digested by N-acetylmuramidase (mutanolysin; Sigma-Aldrich) at 20 μg mL^{-1} and lysozyme at 10 μg mL^{-1} at 37°C for 30 min. The samples were then extracted with RNeasy Mini Kit (Qiagen) and further digested by RNase-free DNase (Promega) to remove trace amounts of contaminated DNA. The isolated RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-rad). cDNA samples were then quantified by real-time PCR using the iQ SYBR green supermix kit (Bio-Rad) (Liu *et al.*, 2011). Primers used for qRT-PCR are listed in Table 2.

Western blotting analysis

The same cell numbers of each bacterial samples were spin down and digested by 100 μL of mutanolysin and lysozyme mixture in lysis buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 0.5% [vol/vol] NP-40) for 10 min at room temperature (Liu *et al.*, 2011). Cell lysates were harvested by centrifugation at 13,000 rpm for 5 min after the removal of cell debris and used as protein extracts. Cell supernatants were harvested and precipitated with 30% ethanol at −80°C for 15 minutes, supernatant proteins were harvested by centrifugation at 13,000 rpm for 15 min at 4°C. Protein samples were then dissolved into 5× SDS loading buffer and subjected to SDS-PAGE analysis, followed by Western blotting using GtfB, antigen I&II, GbpA, GbpD and DnaK antiserum. Western blotting was carried

out using procedures described previously (Liu *et al.*, 2011). Fluorescence intensity was quantified by the method of Gassmann *et al* using Image J 1.48v (Windows version of NIH Image, http://rsb.info.nih.gov/nih-image/) (Gassmann *et al.*, 2009, Tan & Ng, 2008).

Colonization of *Drosophila*

Drosophila were maintained routinely on medium containing corn meal, agar, and sucrose. Infections were performed as described (Chugani *et al.*, 2001, Mulcahy *et al.*, 2011). Mid-log phase THB cultures of *S. mutans* were spun down and resuspended in 5% sucrose. Cultures were adjusted to an OD600=25 (2.5×10^{10} CFU per ml) in sucrose. The resuspended cells (100 μl) were spotted onto a sterile filter (Whatman) that was placed on the surface of 5 ml of solidified 5% sucrose agar in a plastic vial (VWR). The vials were allowed to dry at room temperature for 30 minutes prior to addition of Drosophila. Male Canton S flies (1–3 days old) were treated with antibiotics for 2 days and starved for 3 hours prior to add to vials (10–14 flies per vial). The vials were capped with cotton, inverted over a tray with water for humidity, and incubated at 25°C. Flies were anaesthetized by carbon dioxide throughout the sorting and transferring process. To determine the number of viable bacterial cells associated with individual flies, single flies were ground with tips in an Eppendorf tube with 100 μl of THB, and serial dilutions of the homogenate were spread on THB agar plates. To visualize the colonization of Drosophila by *S. mutans*, we fed flies with gfp-tagged (green fluorescent protein) strains and examined effects of various mutations on bacterial colonization as described (Dionne *et al.*, 2003). The colonization of flies by *gpf*-tagged strains were analyzed by Nikon elipse 90i microscope, equipped with an Epi-fluorescence and NIS elements AR imaging system. Representative images were presented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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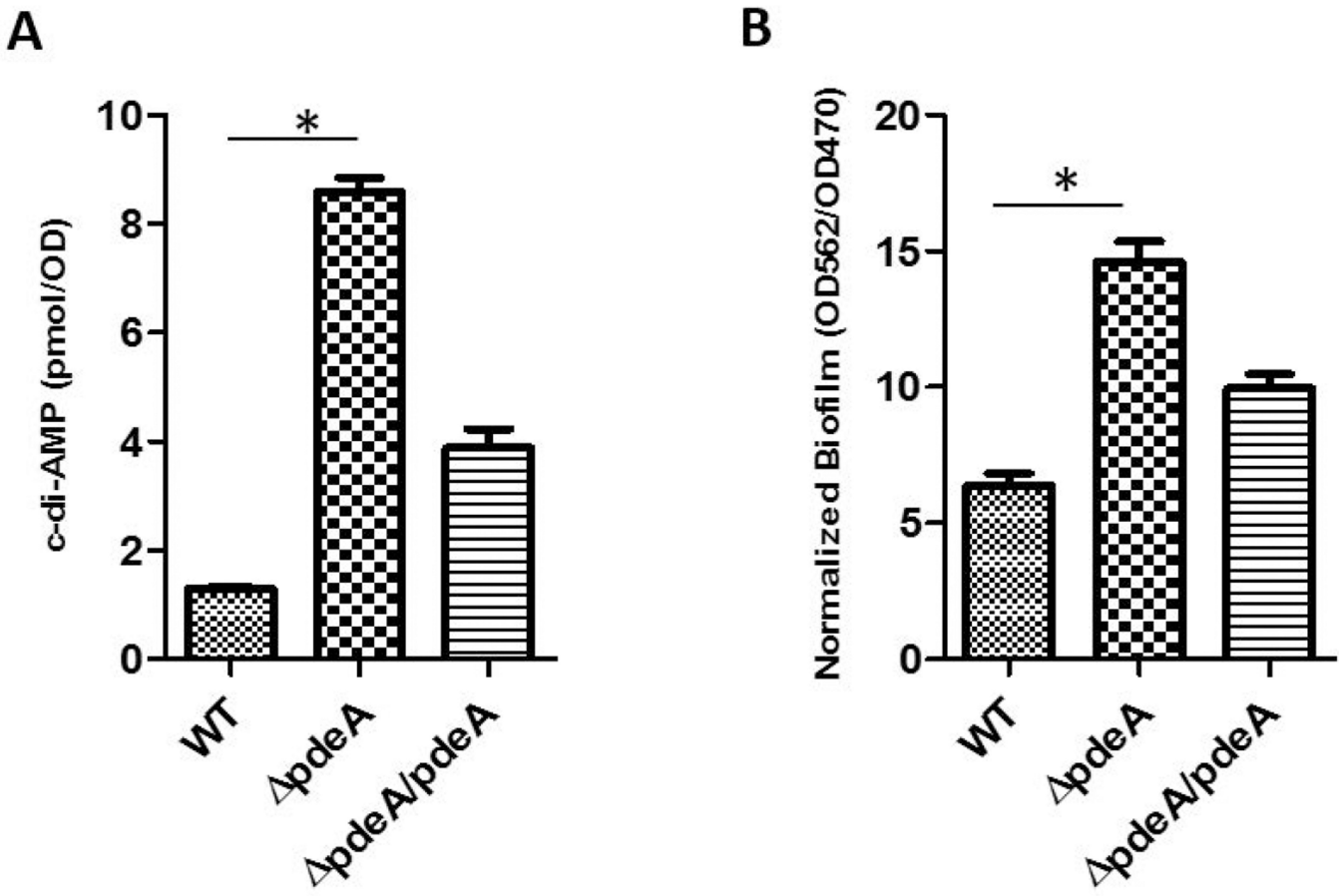
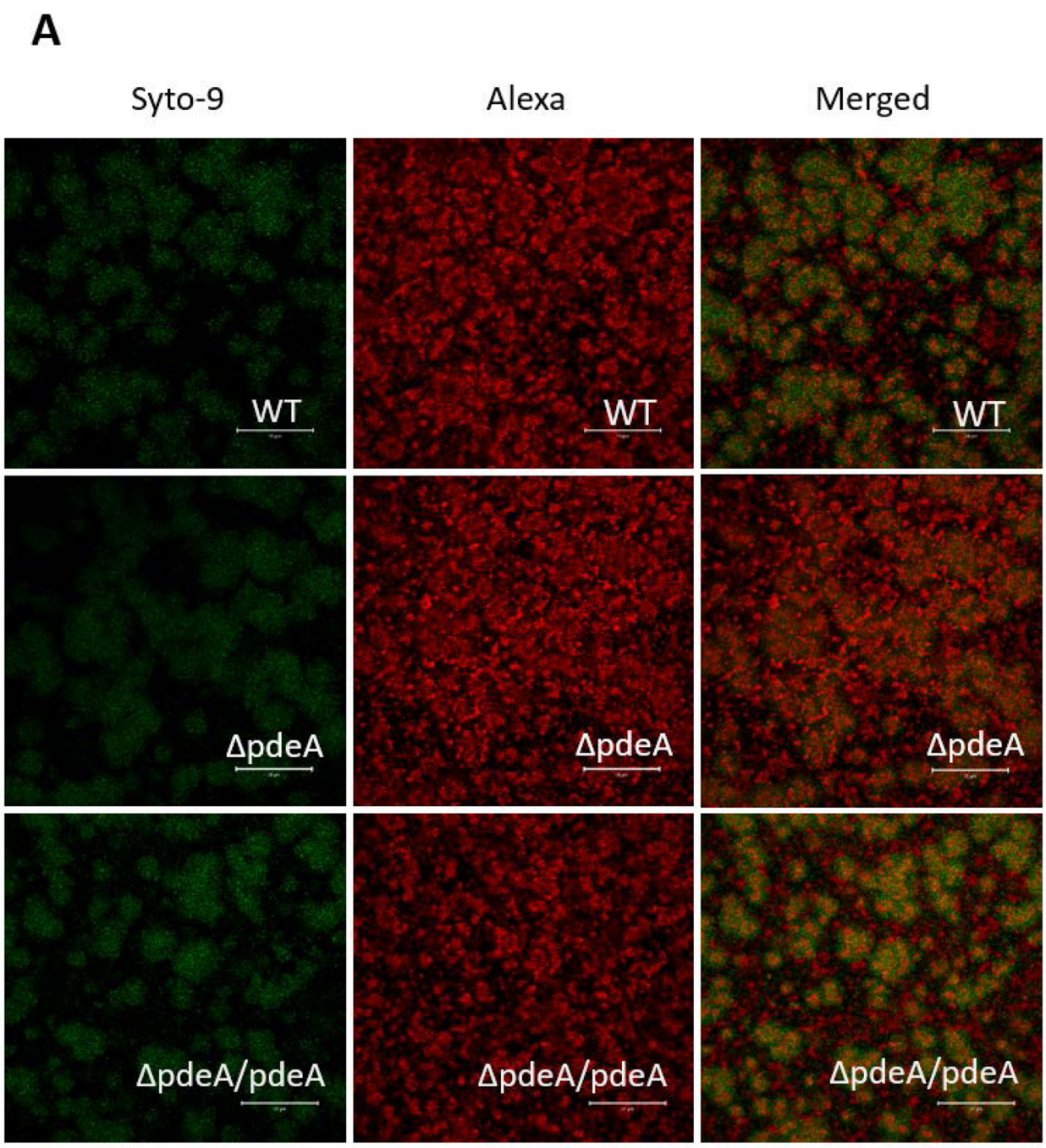


FIG 1. The PdeA deficiency increased c-di-AMP levels and promoted biofilm formation of *S. mutans*

(A) Detection of bacterial c-di-AMP levels. Cell lysates of wild type, the *pdeA* mutant and the complemented strain were used to determine intracellular c-di-AMP levels by competitive ELISA. Data were normalized by bacterial growth OD_{470} of the cultures. (B) Biofilm formation by the *pdeA* mutant. Biofilm mass of wild type, the *pdeA* mutant and the complemented strain were determined using crystal violet staining assay and normalized to bacterial growth by OD_{562}/OD_{470}. Means of three independent experiments are shown. Error bars denote the SEM. *, $P < 0.01$ of Student's *t* test using Prism 5.0.



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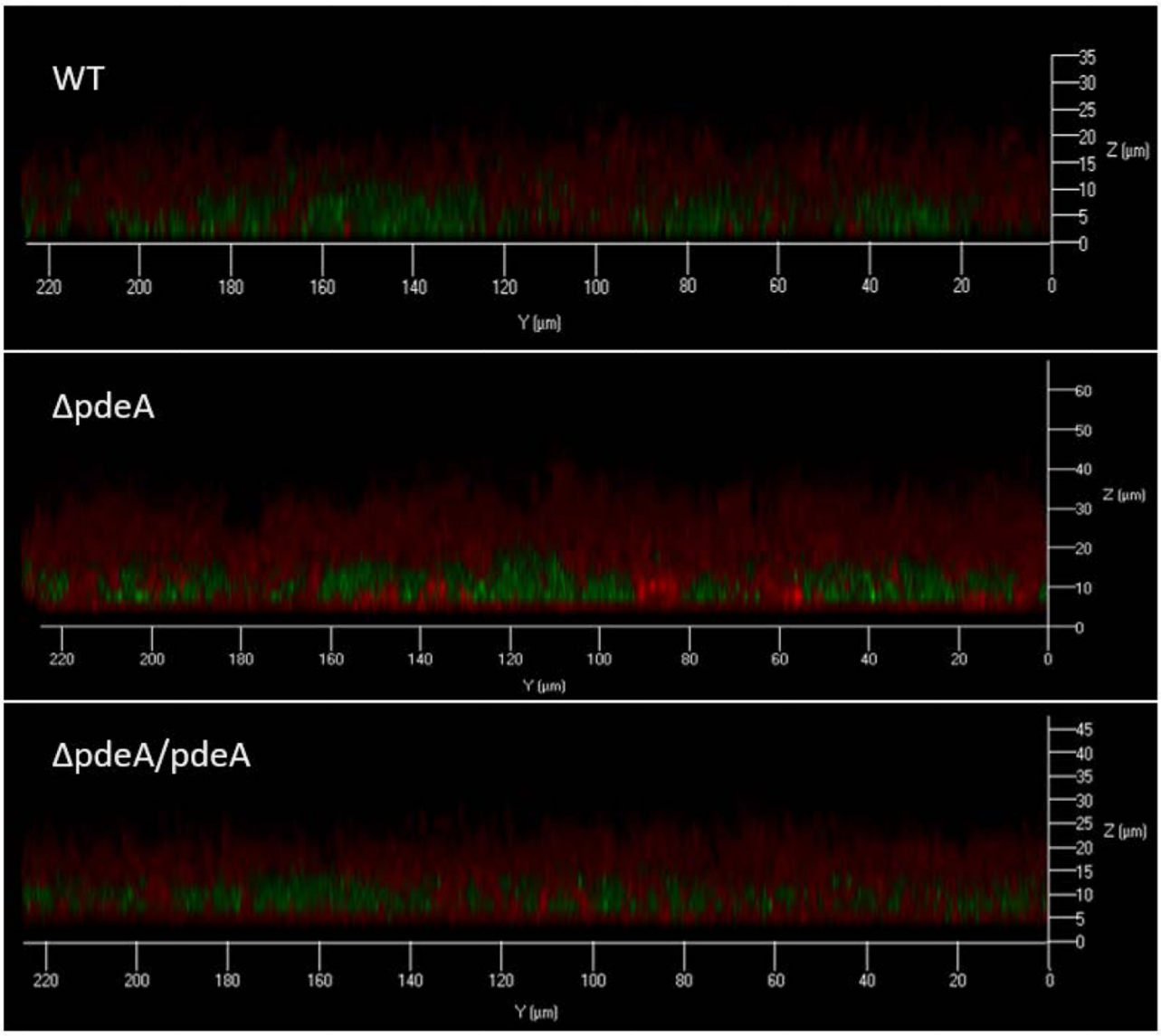


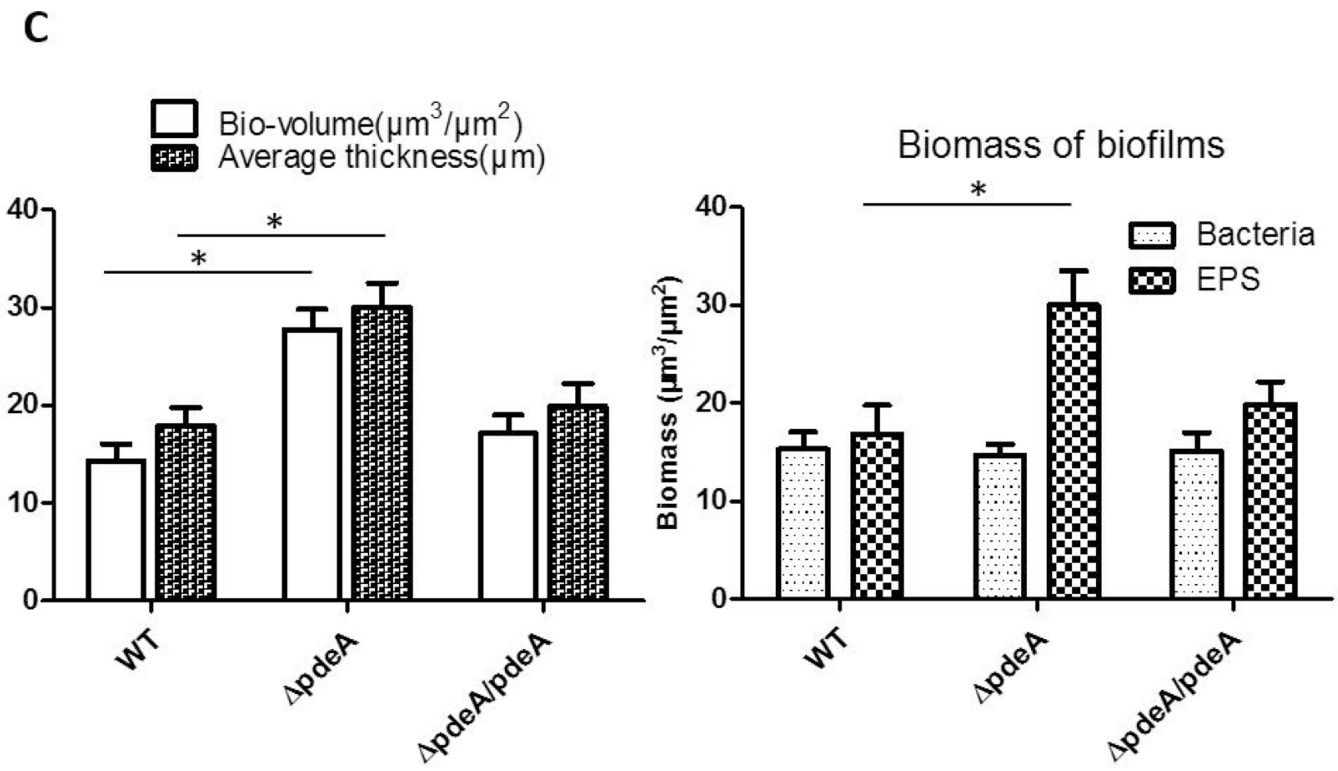
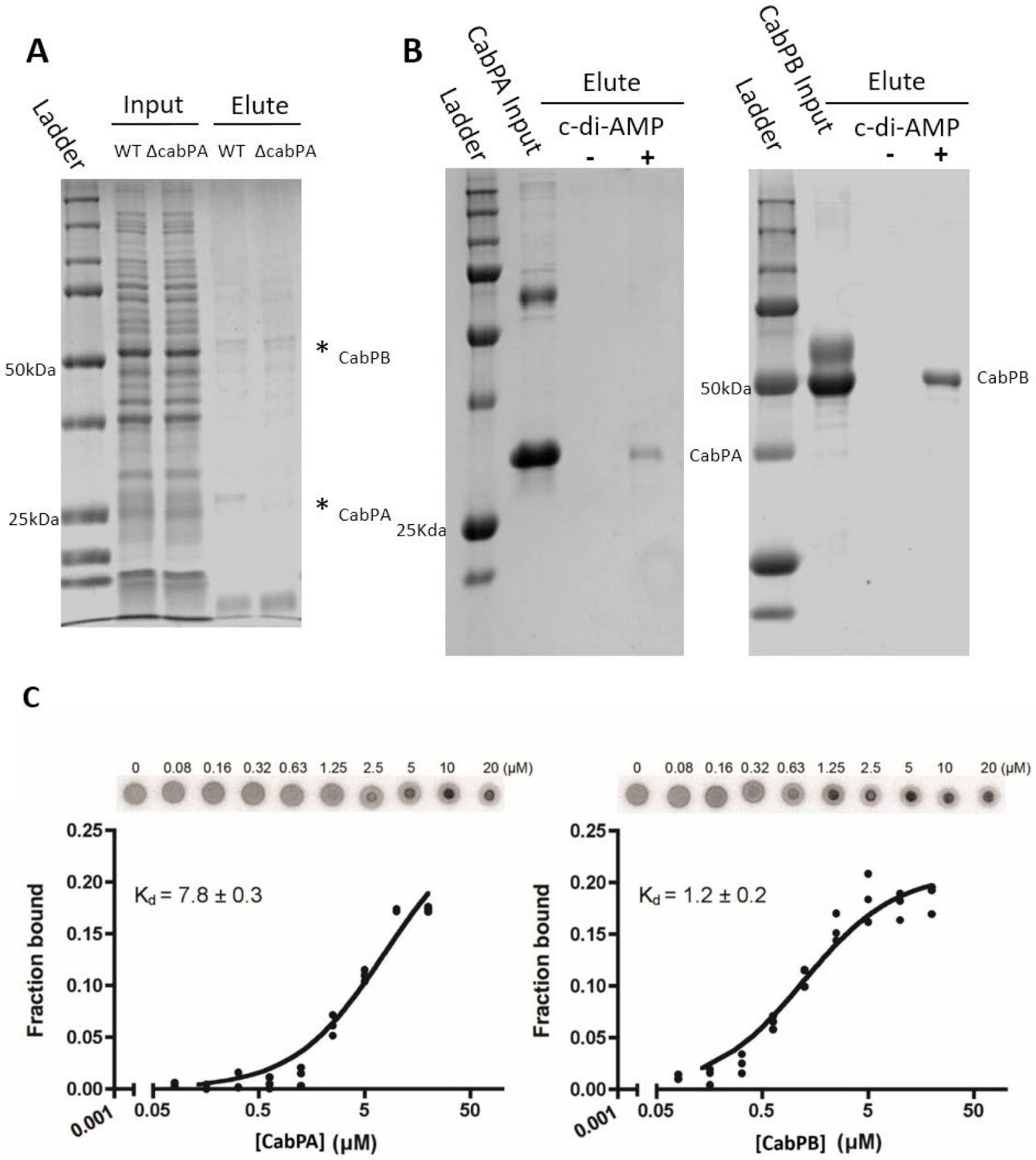


FIG 2. PdeA deficiency-mediated biofilm formation analyzed by confocal laser scanning microscopy (CLSM)

(A) Representative images of 16 hours biofilms of each *S. mutans* strain analyzed using CLSM. The left panels represent bacteria stained by SYTO-9 (green), the middle panels represent EPS stained by dextran-conjugated Alexa Red, and the right panels represent merged images. Representative images from three independent experiments are shown. 50 μm scale bar was showed in each image. (B) Images of vertical sections of biofilms analyzed by CLSM, using CLSM 5 Image Browser software. (C) COMSTAT analysis of bio-volume and depths of biofilm depicted in (B). Total biomass and thickness were analyzed (Left panel); bacteria and EPS biomass were analyzed separately (Right panel). Data shown are the means with standard deviations of results from 6 representative images of each group taken from two difference days by the same person. *, $P < 0.05$ of Student's *t* test using Prism 5.0.



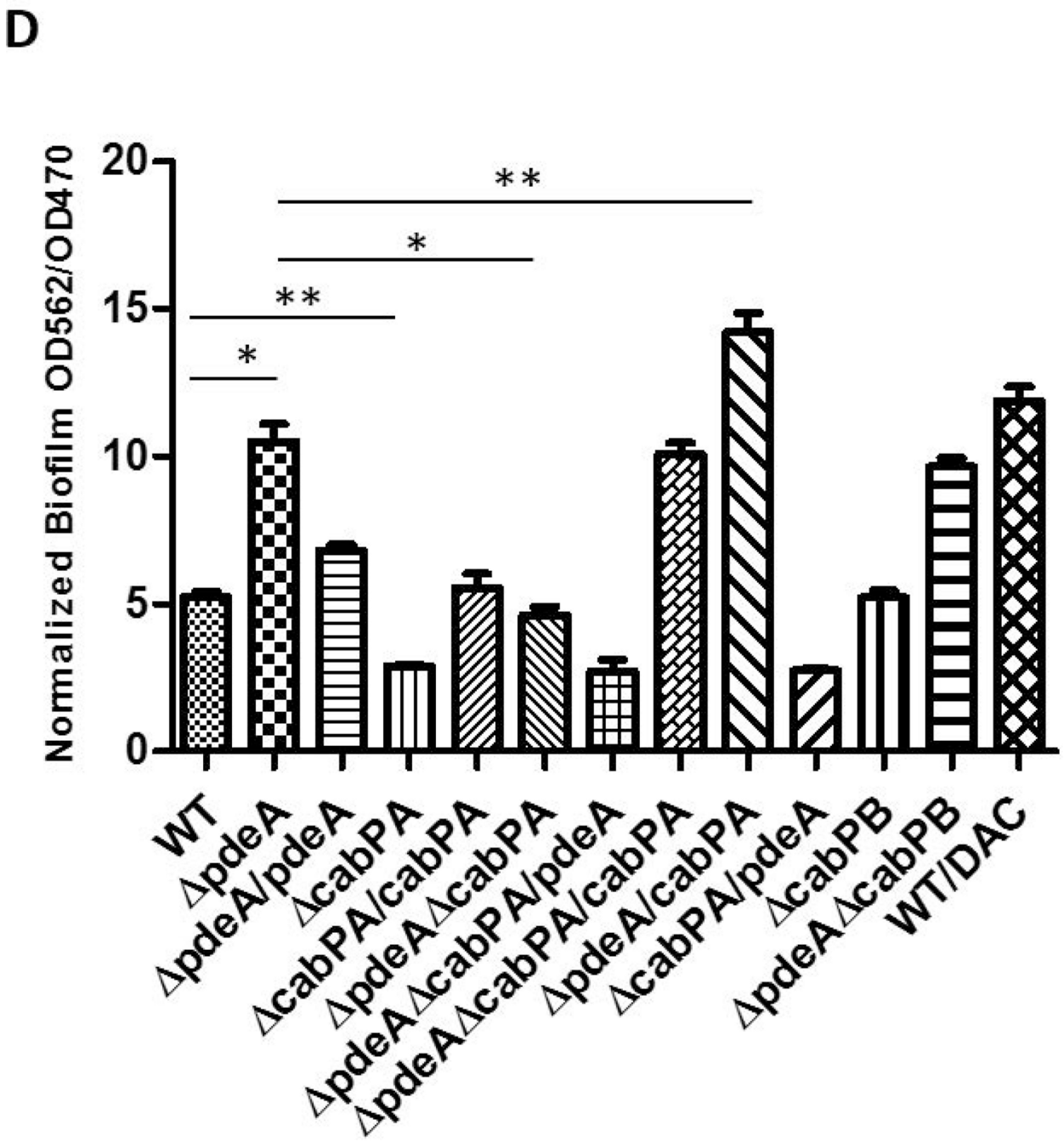


FIG 3. Identification of c-di-AMP binding proteins

(A) Affinity purification of c-di-AMP binding proteins of *S. mutans*. Cell lysates prepared from the *cabPA* mutant and wild type strain of *S. mutans* were allowed to pass through a 2'-AHC-c-di-AMP agarose column. Proteins eluted from the column after three washes were subject to SDS-PAGE analysis and staining. Two protein bands (*) evident in the wild type elution were identified as CabPA and CabPB by LC/MS/MS. (B) Recombinant CabPA and CabPB proteins bind to c-di-AMP. Purified recombinant CabPA (Left panel) and CabPB (Right panel) were used in *in vitro* pull-down assays with c-di-AMP-coupled (+) or

uncoupled (−) beads. BSA was included in the inputs with recombinant CabPA and CabPB as a negative control. Proteins bound to the beads were subject to SDS-PAGE analysis and protein staining. (C) Binding affinity of CabPA and CabPB to c-di-AMP measured by DRaCALA. CabPA had a lower affinity (in μM) binding to c-di-AMP when compared to CabPB. (D) Effects of deletion or overexpression of *cabPA* on biofilm formation by the *pdeA* mutant. Biofilm formation of wild type and different mutant variants and complements were determined using crystal violet staining assay. Means of three independent experiments are shown. Error bars denote the SEM. *, $P < 0.01$; **, $P < 0.05$ of Student's t test using Prism 5.0.

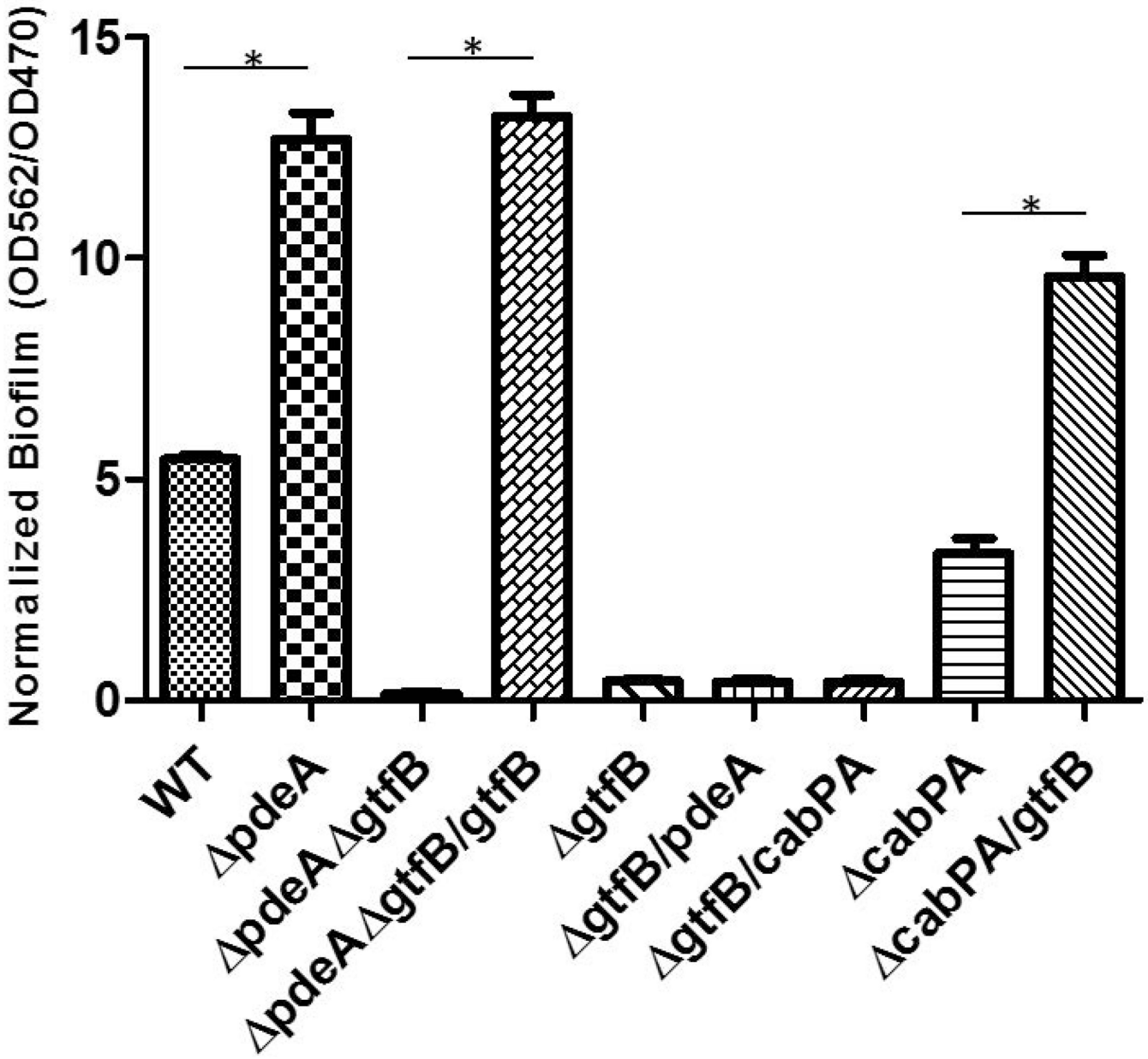
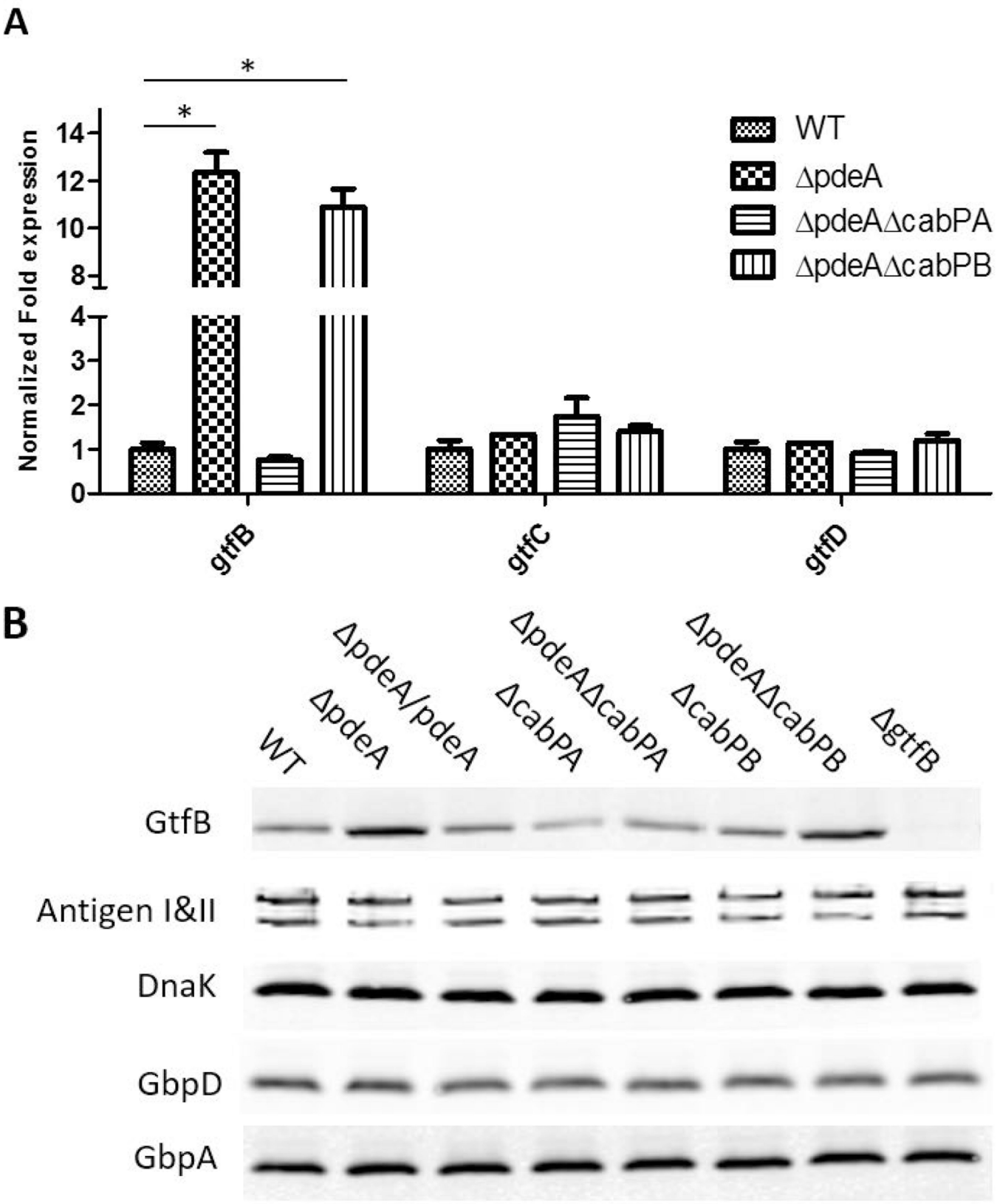


FIG 4. GtfB is crucial for c-di-AMP-mediated biofilm formation

Biofilm deficient mutant *pdeA-gtfB* was identified by screening of a transposon mutant library on the *pdeA* mutant background. Cross-complementation of various mutants by GtfB, CabpA and PdeA was carried out to determine their effects on biofilm formation. Biofilm formation of wild type, the *pdeA* mutant, the identified insertional GtfB mutant and the complemented strain were determined by OD_{562}/OD_{470} using a crystal violet staining assay. Means of three independent experiments are shown. Error bars denote the SEM. *, $P < 0.01$ of Student's *t* test using Prism 5.0.



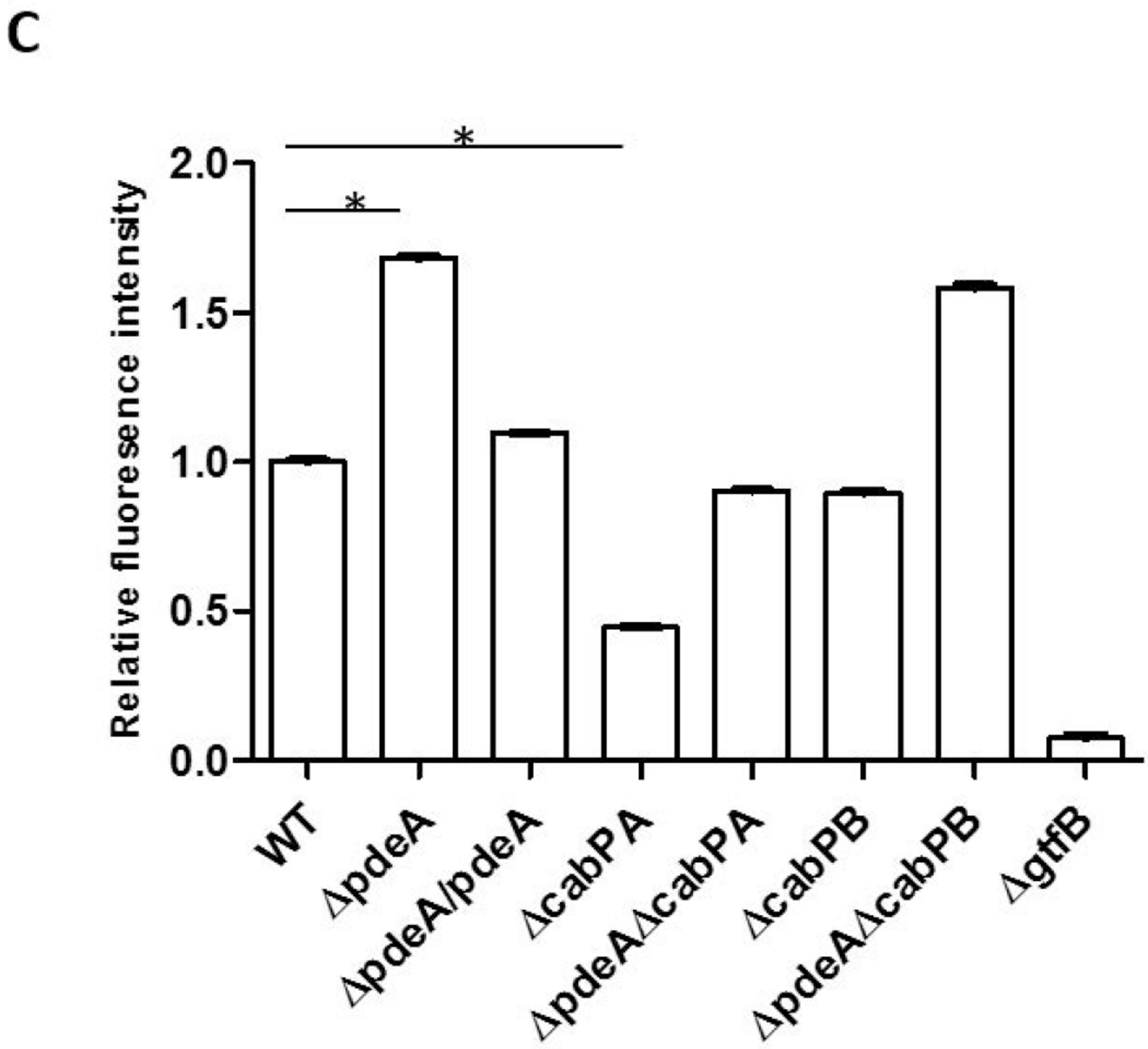


FIG 5. CabPA is critical for the regulation of GtfB

(A) Quantitative analysis of expression of glucosyltransferase GtfBCD genes. Expression of *gtfB, gtfC* and *gtfD* in the indicated mutants was determined by qRT-PCR using RNA prepared from log-phase grown bacteria. Expression of mRNA was calibrated using 16S rRNA. Values represent the means of three different experiments with standard deviations. * = $P < 0.01$ of Student's *t* test using Prism 5.0. (B) Western blotting analysis of GtfB and other *S. mutans* proteins. The amount of GtfB, AntigenI/II, GbpA, and GbpD in different *S. mutans* mutants was examined by Western blotting analysis of cell lysates of 6-h *S. mutans* cultures. Dnak was set as a loading control. (C) Quantification of Western blotting analysis of GtfB. Values represent the means of three experiments with standard deviations of relative

fluorescence intensity of each band analyzed by using ImageJ. * = P < 0.01 of Student's *t* test using Prism 5.0.

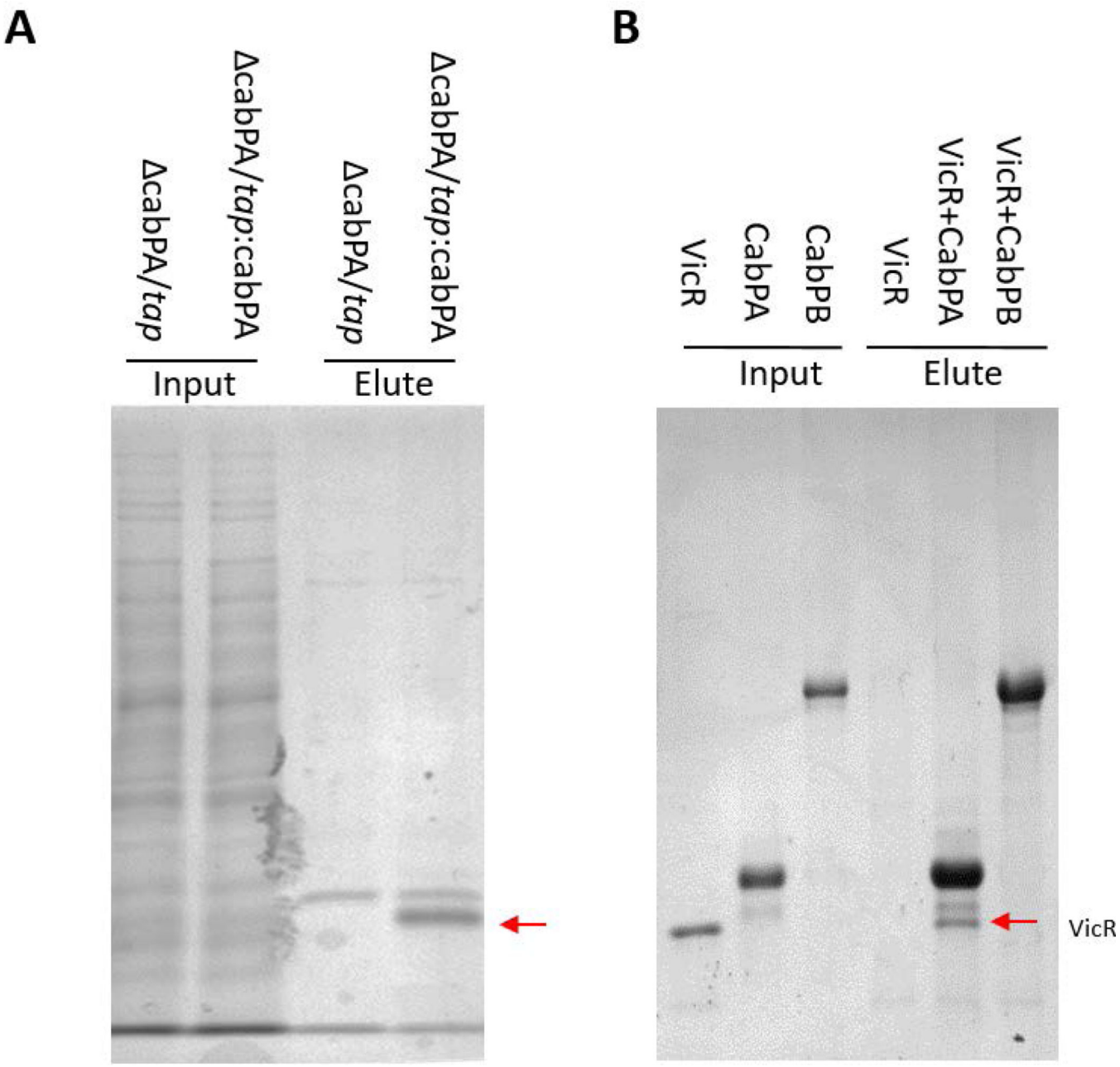


FIG 6. CabPA, not CabPB, interacted with VicR

(A) Coomassie blue staining SDS-PAGE of TAP purification. Both input and elute samples were analyzed. A band at 25 kDa was evident in *tap:cbpA* but not the tap control lane (arrow indicated) and identified as VicR by LC/MS/MS. (B) CabPA not CabPB bound to VicR as determined by a pull-down assay. Recombinant VicR could be pulled down by recombinant CabPA, not CabPB.

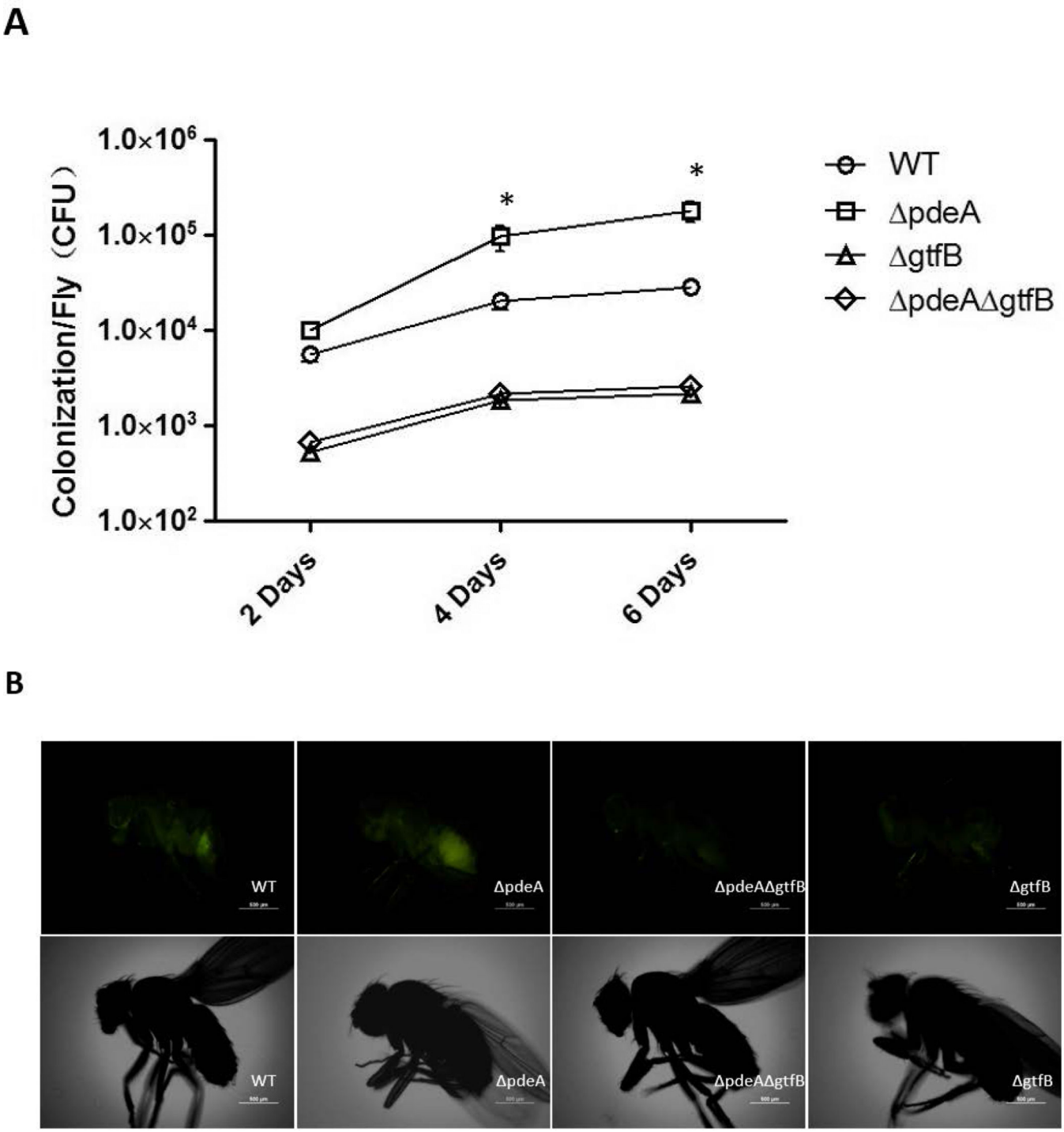


FIG 7. Colonization of *Drosophila*

(A) Quantification of bacterial colonization. Colony forming units/fly at 2, 4 and 6 days after infected with *S. mutans* wild type, PdeA, GtfB and PdeA-GtfB double mutants were enumerated. * = P < 0.01 of Student's *t* test using Prism 5.0. (B) Fluorescent detection of *S. mutans* colonized Drosophila. Representative images of Drosophila colonized by *gfp*-tagged

S. mutans variants on 6 days were analyzed by Nikon elipse 90i microscope, equipped with an Epi-fluorescence and NIS elements AR imaging system.

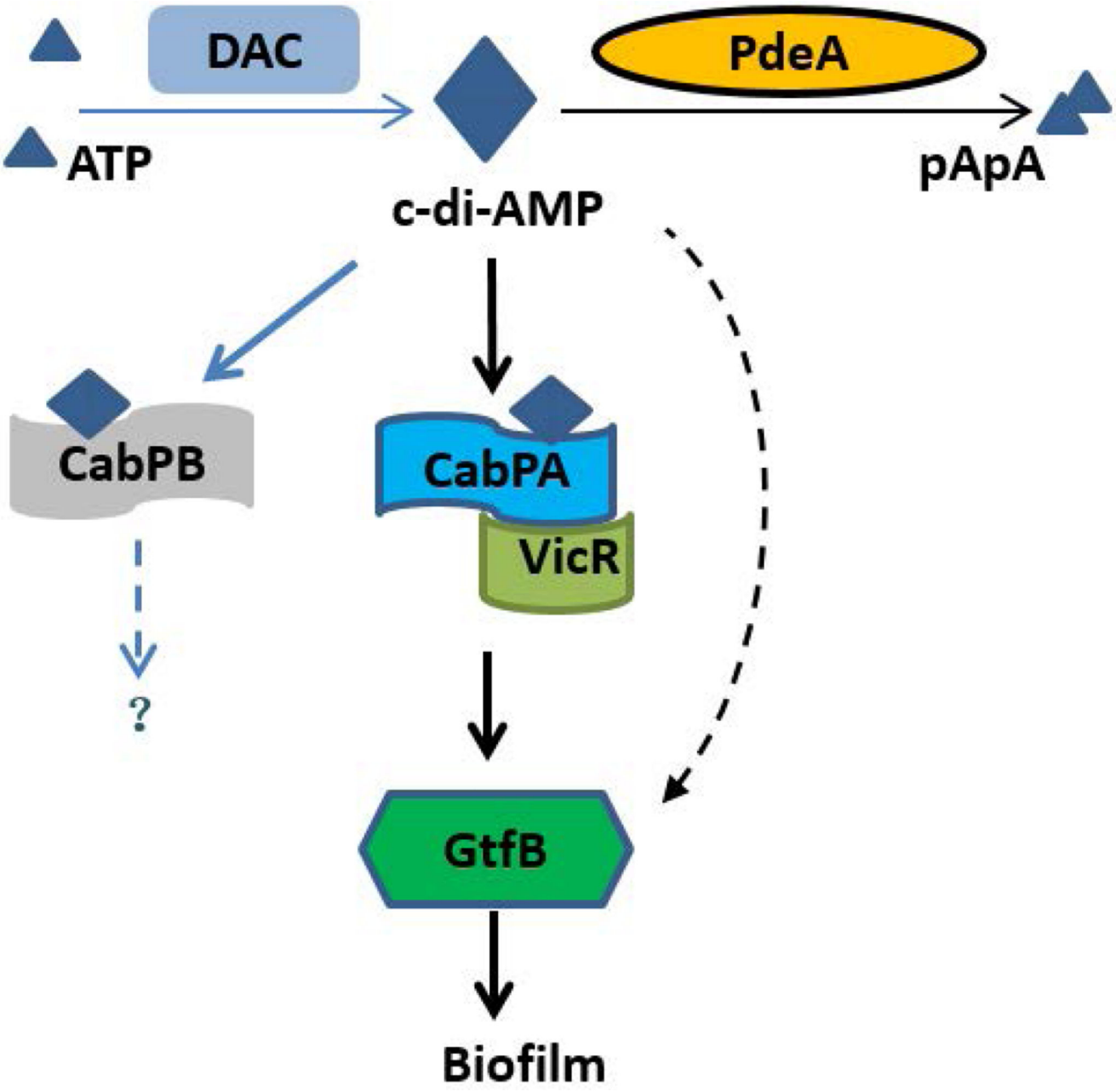


FIG 8. Working model of c-di-AMP-mediated biofilm formation

Elevated c-di-AMP binds to its receptor CabPA, and CabPA in turn interacts with VicR, a response regulator that modulates expression of *gtfB*, thereby promoting biofilm formation. Two homologous c-di-AMP receptors CabPA and CabPB were identified, only CabPA was found to bind to VicR, thereby increasing expression of *gtfB* and promoting biofilm formation. CabPB is not involved in biofilm formation, the precise function of CabPB is unknown. Alternative pathways may exist that engage c-di-AMP receptors other than CabPA and CabPB to regulate GtfB-dependent biofilm formation. Solid lines represent well-supported findings, and broken lines represent hypothetical or unknown functions.

Table 1

Bacterial strains and plasmids used in this study

Strains	Description	Source
S.mutans UA159	Parent strain	(Liu *et al.*, 2011)
ΔpdeA	PdeA deletion; Kan^r	This study
ΔpdeA /pdeA	PdeA complementation; Kan^r; Erm^r	This study
ΔcabPA	CabPA deletion; Erm^r	This study
ΔcabPB	CabPB deletion; Erm^r	This study
ΔpdeAΔcabPA	PdeA and CabPA double mutant; Kan^r; Erm^r	This study
ΔpdeAΔcabPB	PdeA and CabPB double mutant; Kan^r; Erm^r	This study
ΔpdeAΔgtfB	GtfB insertion mutant on PdeA; Kan^r; Spec^r	This study
ΔgtfB	GtfB deletion; Kan^r	This study
ΔcabPA/*tap*	CabPA deletion with *tap*; Kan^r; Erm^r	This study
ΔcabPA/*tap*:cabPA	Recombinant CabPA with *tap*; Kan^r; Erm^r	This study
ΔpdeAΔgtfB/gtfB	Complementation of PdeA-GtfB using GtfB; Erm^r ;Kan^r; Spec^r	This study
WT/DAC	Overexpressed DAC on wild type *S. mutans*	This study
S. mutans/gfp	gfp-tagged *S. mutans* UA159; Kan^r	(Liu *et al.*, 2011)
ΔpdeA/gfp	gfp-tagged PdeA deletion; Kan^r; Erm^r	This study
ΔpdeAΔgtfB/gfp	gfp-tagged PdeA-GtfB double mutant; Kan^r; Erm^r	This study
ΔgtfB/gfp	gfp-tagged GtfB deletion; Kan^r; Erm^r	This study
E.coli pET28b–CabPA	CabPA expression; Kan^r	This study
E.coli pET28b–CabPB	CabPB expression; Kan^r	This study
E.coli pET28a–sumo-VicR	VicR purification; Kan^r	Wu Lab
E. coli Top 10	Cloning strain	Invitrogen
E. coli BL-21	Protein production strain	Invitrogen
Plasmids		
pVPT	*E. coli*-Streptococci shuttle vector; Erm^r	(Zhou *et al.*, 2008)
pVPT-pdeA	pVPT carrying PdeA ORF; Erm^r	This study
pVPT-*tap*	pVPT carrying *tap* tag; Erm^r	Wu Lab
pVPT-*tap*-cabPA	pVPT carrying *tap* tag and CabPA; Erm^r	This study
pET28b(+)	Expression plasmid; Kan^r	Novagen
pET28a–sumo	Expression plasmid; Kan^r	Wu Lab
pET28b–cabPA	pET28b(+) carrying cabPA ORF; Kan^r	This study
pET28b–cabPB	pET28b(+) carrying cabPB ORF; Kan^r	This study

Table 2

Primers used in this study

Primers	Sequence (5'-3')	Description
pdeAupF	TATGATATTGTCCTTCCTCATC	PdeA deletion
pdeAupR	TATTCTCATTTTAGCCATTATCTAAACCTCTTAAGCCCTA	PdeA deletion
pdeAdnF	CTGGATGAATTGTTTTAGGATGAAAGTTATTTTTTTAGCA	PdeA deletion
pdeAdnR	CGAAATCCACTACGATTACT	PdeA deletion
pedAcheckF	ATGTGGTTCATTCGATTAAA	Check PdeA mutant
pdeAcheckR	GAACTTCTAAATTTTCATAATTGAC	Check PdeA mutant
pdeAF	ATGAAAAGATTTCGTTTTGCCAC	Amplify PdeA
pdeAR	TTAAGCTTCCTCGTTTCCTG	Amplify PdeA
kanF	ATGGCTAAAATGAGAATA	Amplify kanamycin
kanR	CTAAAACAATTCATCCAG	Amplify kanamycin
ermF	GGAAGGAGTGATTACATGAA	Amplify rythromycin
ermR	GAAGCTGTCAGTAGTATACC	Amplify rythromycin
cabPAupF	GCCTTATCAGATGAAGTTGTC	CabPA deletion
cabPAupR	TTCATGTAATCACTCCTTCCAGCTACCCTCCTTTTGATAAC	CabPA deletion
cabPAdnF	GGTATACTACTGACAGCTTCGGAGGAAGAAGCTATGAAAAT	CabPA deletion
cabPAdnR	CTACTATCGAGCTCTTCAACTG	CabPA deletion
cabPAcheckF	AAGATATGTCAGATGATGACTTGA	Check cabPA mutant
cabPAcheckR	AGCTGGACTTTTCTAATTCTTTC	Check cabPA mutant
cabPBupF	CCACAGCATAAGTATGACTAA	CabPB deletion
cabPBupR	TTCATGTAATCACTCCTTCC GATTTACCTCCGAATTATAA	CabPB deletion
cabPBdnF	GGTATACTACTGACAGCTTCGCTATTATGAATAAAAGTATGG	CabPB deletion
cabPBdnR	TAAACTGAGAAAAGAGCGGCC	CabPB deletion
cabPBcheckF	TTTTTGTAATACTTTTAACGCATAT	Check cabPB mutant
cabPBcheckR	ACCAAGAAACATCAGTAGAAGC	Check cabPB mutant
cabPAF	GTCAGGATCCG ATGAAAATTATTATTGTCGGATGTGG	CabPA expression
cabPAR	GCCGGAGCTC TCAGAAAATAAAGCGTTCTCTC	CabPA expression
cabPBF	GTCAGGATCCG ATGAAAATTATTGTCGTTGGTGG	CabPB expression
cabPBR	GCCGGTCGAC TTACCTCTTTAATAAATCATAAA	CabPB expression
Q16sRNAF	AGCGTTGTCCGGATTTATTG	qRT-PCR
Q16sRNAR	CTACGCATTTCACCGCTACA	qRT-PCR
QgtfBF	CACTATCGGCGGTTACGAAT	qRT-PCR
QgtfBR	CAATTTGGAGCAAGTCAGCA	qRT-PCR
QgtfCF	GATGCTGCAAACTTCGAACA	qRT-PCR
QgtfCR	TATTGACGCTGCGTTTCTTG	qRT-PCR
QgtfDF	TTGACGGTGTTCGTGTTGAT	qRT-PCR

Primers	Sequence (5'-3')	Description
QgtfDR	AAAGCGATAGGCGCAGTTTA	qRT-PCR
cabPAtapF	GCCGGTCGACATGAAAATTATTATTGTCGGATGTGG	TAP construct
cabPAtapR	GCGCGGTACCTCAGAAAATAAAGCGTTCTCTC	TAP construct