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BACTERIAL INFECTIONS

AtlA Mediates Extracellular DNA Release, Which Contributes to *Streptococcus mutans* Biofilm Formation in an Experimental Rat Model of Infective Endocarditis

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ABSTRACT Host factors, such as platelets, have been shown to enhance biofilm formation by oral commensal streptococci, inducing infective endocarditis (IE), but how bacterial components contribute to biofilm formation *in vivo* is still not clear. We demonstrated previously that an isogenic mutant strain of *Streptococcus mutans* deficient in autolysin AtlA (Δ*atlA*) showed a reduced ability to cause vegetation in a rat model of bacterial endocarditis. However, the role of AtlA in bacterial biofilm formation is unclear. In this study, confocal laser scanning microscopy analysis showed that extracellular DNA (eDNA) was embedded in *S. mutans* GS5 floes during biofilm formation on damaged heart valves, but an Δ*atlA* strain could not form bacterial aggregates. Semiquantification of eDNA by PCR with bacterial 16S rRNA primers demonstrated that the Δ*atlA* mutant strain produced dramatically less eDNA than the wild type. Similar results were observed with *in vitro* biofilm models. The addition of polyanethol sulfonate, a chemical lysis inhibitor, revealed that eDNA release mediated by bacterial cell lysis is required for biofilm initiation and maturation in the wild-type strain. Supplementation of cultures with calcium ions reduced wild-type growth but increased eDNA release and biofilm mass. The effect of calcium ions on biofilm formation was abolished in Δ*atlA* cultures and by the addition of polyanethol sulfonate. The VicK sensor, but not CiaH, was found to be required for the induction of eDNA release or the stimulation of biofilm formation by calcium ions. These data suggest that calcium ion-regulated AtlA maturation mediates the release of eDNA by *S. mutans*, which contributes to biofilm formation in infective endocarditis.

KEYWORDS *Streptococcus mutans*, biofilm, extracellular DNA, infective endocarditis

In addition to forming planktonic populations, bacteria can colonize and accumulate on surfaces to form biofilms, which are crucial in the pathogenesis of many subacute and chronic bacterial infections (1, 2). A typical example of a biofilm-associated disease is infective endocarditis (IE), which is most frequently caused by staphylococci or streptococci (3, 4). In particular, a population-based cohort study indicated that viridans group streptococci continue to outnumber *Staphylococcus aureus* as the most common organisms causing IE in the general population (5, 6). Our previous report showed that *Streptococcus mutans* and other commensal viridans group streptococci form biofilms on damaged heart valves. These biofilms were composed of layers of bacterial floes

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enclosed in activated platelet aggregates (7), but the detailed composition of the matrix inside the bacterial floes is not clear.

Bacterial biofilms are characterized by polymicrobial aggregates in the form of mats or floes and are typically encased in extracellular polymeric substances (3, 8, 9). Extracellular polymeric substances consist of a wide variety of polysaccharides, proteins, glycoproteins, glycolipids, and, in many cases, extracellular DNA (eDNA) (9–11). Although polysaccharides and proteins are important components, the role of eDNA as a critical element of the matrix, providing structural stability and protection against antimicrobial agents, has been increasingly recognized (12–15). eDNA was first shown to be present in the extracellular matrix of biofilms formed by *Pseudomonas aeruginosa* (16), but numerous studies since have reported similar eDNA properties in biofilms formed by a wide array of Gram-positive and -negative bacteria (14, 15, 17–19), suggesting that bacterial biofilm stabilization by eDNA is widespread.

eDNA release is typically a consequence of cell lysis. Bacterial murein hydrolases (bacterial autolysin) trigger autolytic cell wall digestion, leading to the release of DNA and other cellular contents into the extracellular environment (20). Bacterial autolysin has been implicated in biofilm formation, apparently by mediating bacterial lysis, with consequent release of eDNA, in diverse species, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis* (14, 21, 22). Studies conducted on *S. mutans* have also demonstrated the roles of its autolysin protein AtlA in biofilm formation on polystyrene surfaces *in vitro* (23). In contrast to sucrose-mediated glucans, which are crucial for *S. mutans* biofilm formation on polystyrene and dental surfaces, the composition of intravascular *S. mutans* biofilm matrices is unclear. Therefore, the current study investigated the role of AtlA in *S. mutans* biofilm formation on the damaged heart valves of rats in a model of IE.

RESULTS

***S. mutans* eDNA mediates biofilm formation on damaged heart valves *in vivo*.** To address the role of eDNA in biofilm formation in IE, green fluorescent protein (GFP)-tagged *S. mutans* GS5 and *atlA*-deficient (Δ*atlA*) mutant strains were intravenously administered to rats with experimental IE. The vegetations were harvested and were analyzed by confocal laser scanning microscopy (CLSM). Detection of eDNA by staining with propidium iodide (PI) suggested that the eDNA was embedded inside *S. mutans* GS5 bacterial aggregates. In contrast, the Δ*atlA* strain appeared as single populations without embedded eDNA (Fig. 1A). To further confirm the existence of bacterial eDNA, total DNA was extracted from the harvested vegetations without breaking the bacteria. The eDNA was semiquantified by PCR with bacterial 16S rRNA primers. In agreement with the CLSM analysis, the level of eDNA detected in the Δ*atlA* sample was much lower than that in the parental wild-type strain (Fig. 1B and C). These data suggest that eDNA release mediated by AtlA contributes to *S. mutans* biofilm formation on damaged heart valves in rats with IE.

AtlA mediates cell lysis and the release of bacterial eDNA. To confirm the role of bacterial eDNA, *S. mutans* biofilms were cultured in the defined culture medium without sucrose (24, 25). *S. mutans* biofilms formed aggregates in which eDNA was embedded, similar to those observed *in vivo* (Fig. 2A, left). Digestion of eDNA with DNase I dramatically reduced biofilm formation (Fig. 2A, center, and B), confirming the role of eDNA in *S. mutans* biofilm formation. In agreement with the *in vivo* data, the Δ*atlA* mutant strain exhibited a reduced ability to form biofilms and appeared as single populations without bacterial aggregates (Fig. 2A, right). Using bacterial 16S rRNA primers, we confirmed the lower level of eDNA release in the Δ*atlA* strain culture supernatant than in the wild-type and complemented (compΔ*atlA*) strains (Fig. 2C). The addition of eDNA purified from the *S. mutans* GS5 culture supernatant restored the biofilm formation ability of the Δ*atlA* mutant strain (Fig. 2D), confirming the role of eDNA in *S. mutans* biofilm formation in the absence of sucrose.

eDNA is required for *S. mutans* biofilm adhesion and development. To further address the roles of bacterial autolysis and eDNA in biofilm development, polyanethol

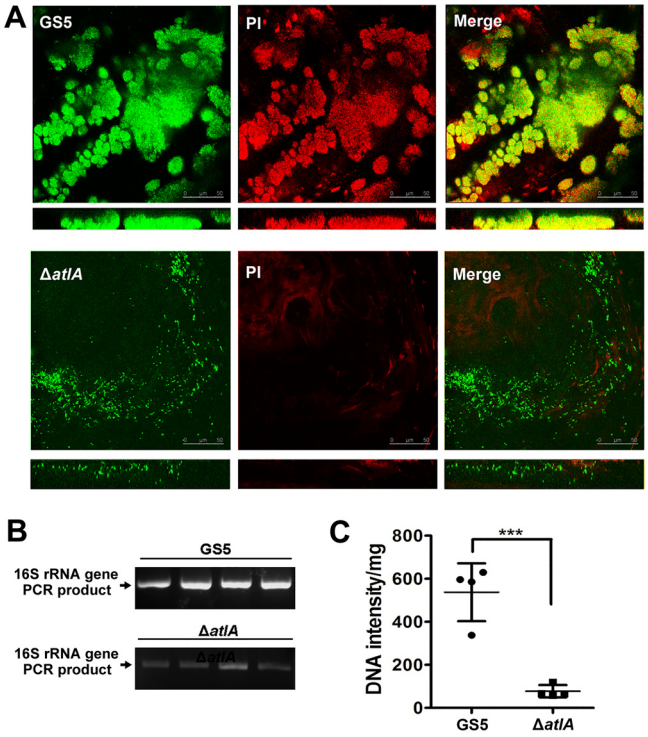

FIG 1 AtlA mediates eDNA release, contributing to *S. mutans* biofilm formation *in vivo*. (A) GFP-tagged wild-type (top) and Δ*atlA* (bottom) biofilms on vegetations harvested from injured rat heart valves were stained with 10 μM PI and were observed by CLSM (magnification, $\times$630). Yellow areas in merged images indicate the presence of both *S. mutans* and eDNA. These experiments were replicated three times, and results of a representative experiment are shown. (B and C) Total DNA isolated from vegetations (without breaking bacteria) was PCR amplified with bacterial 16S rRNA primers, and products were resolved on 1% agarose gels (B) and were quantified with ImageJ software (National Institutes of Health) (C). Data are means $\pm$ standard deviations from four experiments. Asterisks indicate significance (***, $P \leq 0.001$) by Student's *t* test.

sulfonate (PAS), a chemical lysis inhibitor, was used (26). The addition of PAS in the initial stages of biofilm formation abolished eDNA release into the culture medium and inhibited biofilm formation without affecting bacterial growth (Fig. 3A and B; Fig. 3C, 0-h time point). In addition, PAS treatment at the 2-, 4-, 6-, and 8-h time points resulted in dramatic reductions in the amount of adherent biomass; treatment after the biofilm was formed (24-h time point) had no effect (Fig. 3C). These data indicate that eDNA released by cell lysis is necessary at both the initial stage of biofilm adherence and the later stages of biofilm development.

Calcium ions enhance *S. mutans* eDNA release and biofilm formation. Our previous study reported that calcium ions in plasma can enhance the process of AtlA maturation, which modulates *S. mutans* autolytic activity (25). Therefore, the effects of calcium ions on eDNA release and biofilm formation were examined in the present study. The addition of various concentrations of calcium ions to the culture medium of *S. mutans* GS5 enhanced eDNA release and biofilm formation (Fig. 4A, B, and C). Interestingly, the addition of calcium ions also reduced bacterial growth in a dose-dependent manner (Fig. 4A). The enhancing effect of calcium ions on *S. mutans* biofilm formation was not observed with the Δ*atlA* strain but reappeared with the compΔ*atlA* strain (Fig. 2D). CLSM imaging also showed that the addition of calcium ions enhanced the amount of eDNA embedded in bacterial biofilms and increased biofilm formation (Fig. 4D). The enhancement of eDNA release and biofilm formation by calcium ions was also consistently inhibited by the addition of PAS (Fig. 4E and F). Taken together, these data indicate that calcium ions enhance *S. mutans* cell lysis, which increases eDNA release and subsequent biofilm formation.

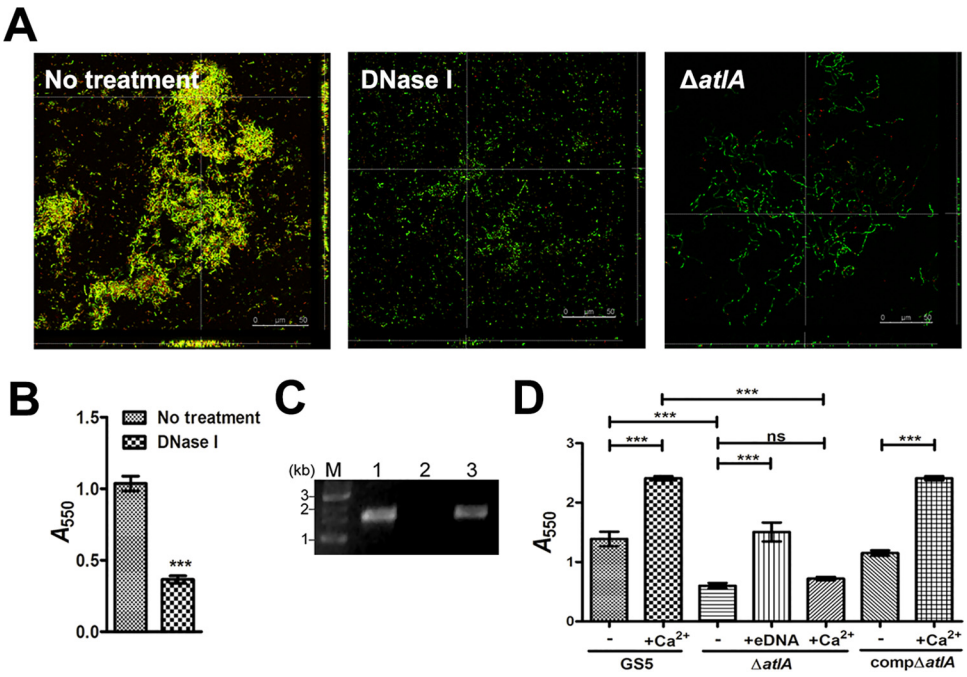

FIG 2 AtlA mediates bacterial eDNA release and biofilm formation. (A) CLSM images of GFP-tagged wild type *S. mutans* biofilms grown without (left) and with (center) DNase I and a Δ*atlA* biofilm (right) stained with 10 μM PI (magnification, ×630). Yellow areas indicate the presence of both *S. mutans* and eDNA. (B) Biofilms grown in 96-well plates with or without DNase I were stained with 0.1% crystal violet. Staining was detected by measuring the absorbance at 550 nm. Data are means ± standard deviations from triplicate experiments. Asterisks indicate significance (***, $P < 0.001$) by Student's *t* test. (C) eDNA released by wild-type (lane 1), Δ*atlA* (lane 2), and compΔ*atlA* (lane 3) strains was amplified by PCR using bacterial 16S rRNA primers, and the products were resolved on 1% agarose gels. M, marker. (D) Biofilms of wild-type, Δ*atlA*, and compΔ*atlA* strains were grown with or without purified bacterial eDNA or 0.1 mM calcium ions and were stained with 0.1% crystal violet. Staining was quantified by measuring the absorbance at 550 nm. Data are means ± standard deviations from triplicate experiments. Asterisks indicate significance (***, $P \leq 0.001$) by one-way ANOVA. These experiments were repeated three times, and results of a representative experiment are shown.

VicK mediates calcium ion-enhanced AtlA maturation and biofilm formation. In *S. mutans*, 14 pairs of two-component systems (TCSs) have been identified by genomic analysis (27). Previous studies have reported that *vicK* disruption leads to inhibition of the N-terminal processing of AtlA (28) and that CiaH is responsible for calcium-mediated regulation in *S. mutans* (29). To investigate which TCS is responsible for sensing calcium ions and mediating eDNA release for biofilm formation, all of the isogenic mutants defective in any of the 14 TCSs were characterized except for the lethal mutant (data not shown). Consistently, the calcium ion-enhanced N-terminal processing of AtlA was abolished in a *vicK*-deficient (Δ*vicK*) mutant strain (Fig. 5A). The effects of calcium ions on eDNA release and biofilm formation were also abolished in the Δ*vicK* strain, but not in the *ciaH*-deficient (Δ*ciaH*) strain, and the addition of bacterial eDNA to the culture medium enhanced biofilm formation by the Δ*vicK* strain (Fig. 5B and C). These results suggest a role for *vicK* in calcium ion sensing and in AtlA-mediated eDNA release and biofilm formation. Furthermore, the Δ*vicK* strain showed a reduced ability to colonize damaged heart valves in an experimental rat model of IE (Fig. 5D and E). Complementation with *vicK* restored all phenotypes of the Δ*vicK* strain (Fig. 5), confirming the role of *vicK*. These data suggest that VicK is responsible for sensing calcium ions in plasma and enhancing the ability of bacteria to form biofilms on damaged heart valves in IE *in vivo*.

DISCUSSION

The intravascular environment is very different from dental surfaces as a place in which to form bacterial biofilms, because it is filled with immune cells and extracellular matrix proteins but does not contain sucrose. Glucosyltransferase-deficient mutant

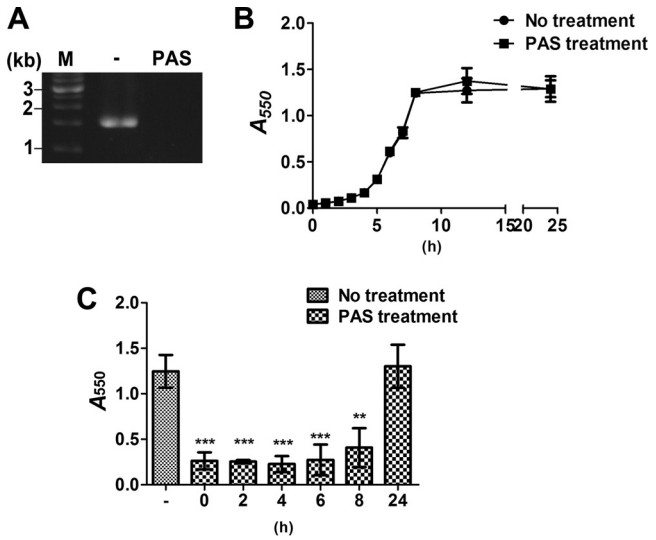

FIG 3 Inhibition of bacterial cell lysis by PAS reduces bacterial eDNA release and biofilm development. (A) eDNA released into the medium of *S. mutans* cultures with or without PAS was detected by PCR amplification using bacterial 16S rRNA primers and resolution of products on 1% agarose gels. M, Marker. (B) The growth curves of *S. mutans* cultured with or without PAS were measured by determining the optical density at 550 nm. (C) *S. mutans* cultures were treated with PAS at the time of inoculation ($t = 0$) and 2, 4, 6, 8, and 24 h postinoculation. All biofilms were grown at 37°C for a total of 24 h. Twenty-four-hour biofilms were allowed to grow for 3 h after the addition of PAS to allow full penetration and activity of the compound on the biofilm. The biofilms were stained with 0.1% crystal violet and were quantified by measuring the absorbance at 550 nm. Data are means ± standard deviations from triplicate experiments. Asterisks indicate significance (***, $P \leq 0.001$; **, $P \leq 0.005$) by one-way ANOVA. These experiments were repeated three times, and results of a representative experiment are shown.

strains, which cannot produce glucans, are still able to cause vegetation formation in the streptococcal IE model, suggesting a distinct matrix composition in *S. mutans* biofilms on the heart valve. While our previous study demonstrated that *S. mutans* biofilms that formed on damaged heart valves *in vitro* and in *in vivo* IE models were composed of bacterial floes enclosed in activated platelet aggregates (7), the matrix composition inside the bacterial floes remains unclear. The present study showed that the autolytic activity of AtlA mediated biofilm formation by *S. mutans* and the embedding of bacterial eDNA in the biofilms. In addition, we previously demonstrated the roles of neutrophil extracellular traps (NETs) in promoting vegetation maturation (30). In contrast to NETs, bacterial eDNA is embedded inside the bacterial aggregates and plays a role in connecting bacteria to each other, contributing to biofilm formation. The NETs formed inside the vegetation cover the bacterial biofilm and have the ability to induce thrombus formation, resulting in vegetation expansion and maturation (30). This may explain why DNase I treatment efficiently reduces both vegetation size and bacterial colony numbers in *S. mutans*-induced IE in rats (30).

In addition to biofilm formation, AtlA also play roles in modulating bacterial resistance to phagocytosis by binding to soluble fibronectin in the circulation (25). AtlA is able to bind various extracellular matrix proteins, including fibrinogen and collagen (data not shown), and may contribute to the ability of the bacteria to bind deposited platelets or exposed collagen on damaged heart valves. AtlA also contributes to *S. mutans* cell division and survival in the circulation (25). Therefore, AtlA plays multiple roles in the pathogenesis of *S. mutans*-induced IE.

Calcium-mediated N-terminal processing of AtlA results in its maturation, which enhances bacterial ability to bind soluble fibronectin in the circulation (25). It also enhances bacterial autolysis, which contributes to bacterial eDNA release and biofilm formation. More interestingly, enhanced bacterial autolysis results in increased bacterial death via "suicidal" or "fratricidal" mechanisms during biofilm development (21, 31). Although bacterial growth conditions would limit biofilm formation, our data showed

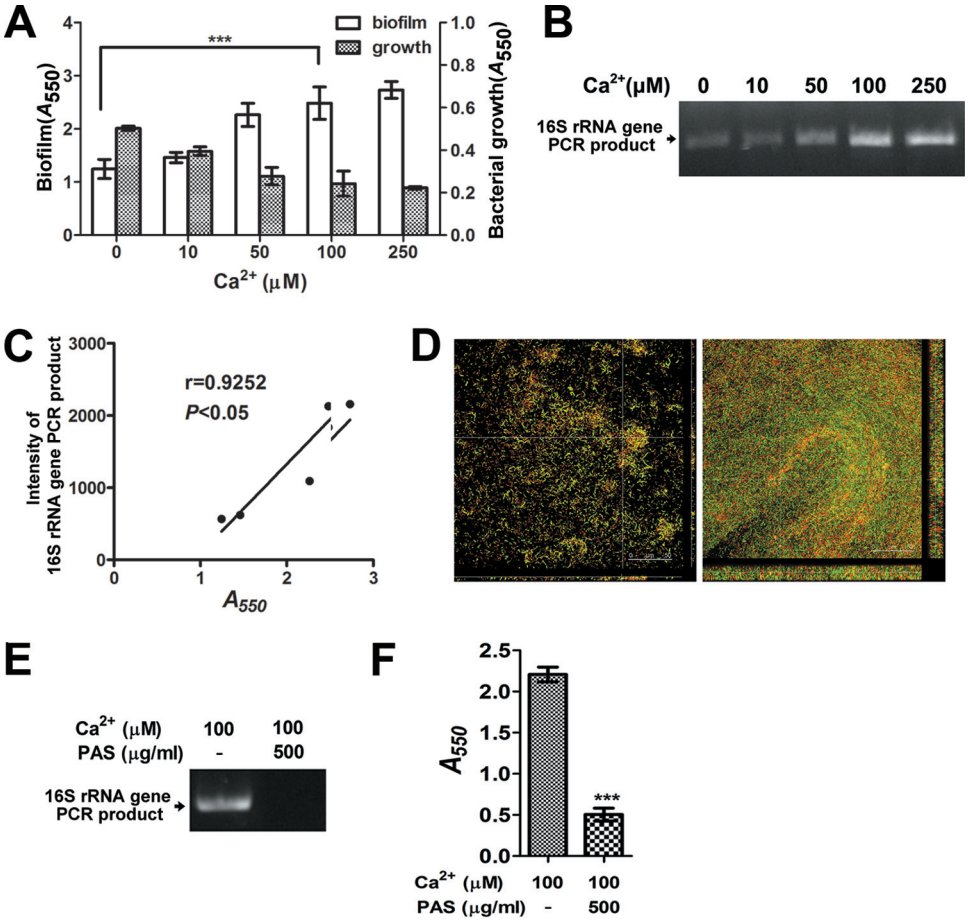

FIG 4 Calcium ions enhance eDNA release and biofilm formation by *S. mutans*. (A) *S. mutans* biofilms grown with a variety of calcium ion concentrations were stained with 0.1% crystal violet and were quantified by measuring the absorbance at 550 nm. Overnight bacterial growth was also measured by determining the optical density at 550 nm. Data are means ± standard deviations from triplicate experiments. Asterisks indicate significance (***, $P < 0.001$) by one-way ANOVA. (B) eDNA released into the medium of *S. mutans* cultures without calcium ions or with different calcium ion concentrations was PCR amplified with bacterial 16S rRNA primers, and the products were resolved on 1% agarose gels. (C) The intensities of 16S rRNA gene PCR products were quantified by ImageJ software, and the correlation between DNA intensity and biofilms was further analyzed by a Pearson correlation test. (D) CLSM images represent *S. mutans* biofilms grown with (right) and without (left) 100 mM calcium (×630 magnification). *S. mutans* was labeled with GFP (green), and bacterial eDNA was stained with 10 μM PI. (E and F) *S. mutans* was cultured with 0.1 mM calcium, with or without PAS. (E) eDNA released into the medium was detected by PCR. (F) Biofilms were stained with 0.1% crystal violet and the absorbance quantified at 550 nm. Data are means ± standard deviations from triplicate experiments. Asterisks indicate significance (***. $P < 0.001$) by Student's *t* test. These experiments were repeated three times, and results of a representative experiment are shown.

that the intensity of the 16S rRNA gene PCR product is statistically correlated with biofilm formation (Fig. 4C), confirming that eDNA mediates biofilm formation. The N-terminal processing of AtlA is mediated by unknown bacterial protease machinery (25). The current results demonstrate that VicK plays a role in calcium ion-enhanced AtlA maturation and biofilm formation (Fig. 5). VicK has also been reported to have global roles in regulating *S. mutans* virulence, including acid tolerance, genetic competence, and oxidative stress tolerance (32–34). In addition, VicRK and CovR coordinate cell division and surface biogenesis with the extracellular synthesis of polysaccharides, a process required for the formation of structurally stable biofilms in the presence of sucrose (35). The current results also demonstrate the role of VicK in eDNA-dependent *S. mutans* biofilm formation in the absence of sucrose. Furthermore, the Δ*vicK* strain showed less ability to form biofilms in the medium without the addition of calcium ions than the wild-type strain (Fig. 5A), suggesting the involvement of other VicK-controlled factors in the pathogenesis of *S. mutans*-induced IE.

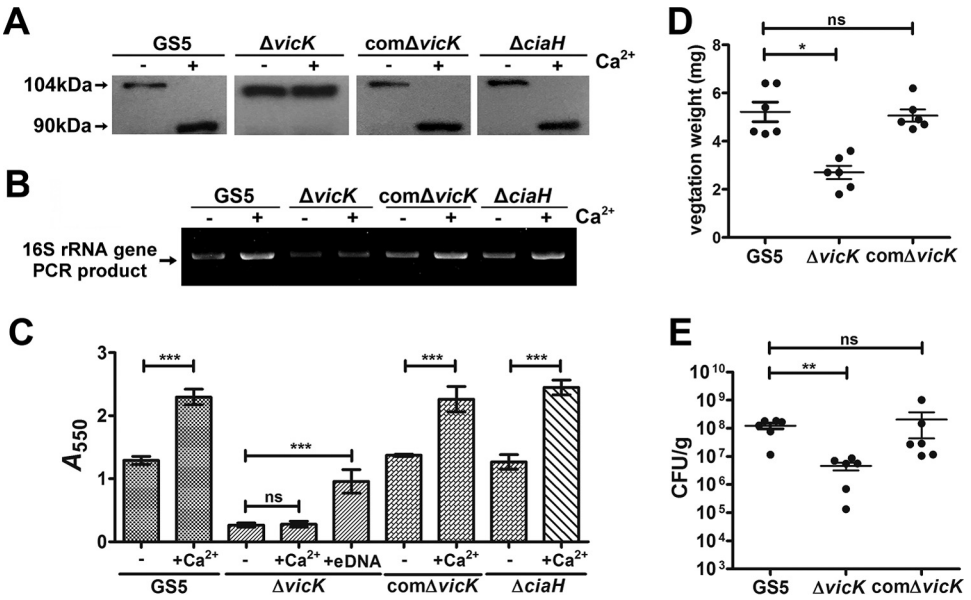

FIG 5 VicK, but not CiaH, is required for calcium ion sensing, induction of AtlA maturation, bacterial eDNA release, and enhanced biofilm formation. (A) *S. mutans* GS5, Δ*vicK*, com*ΔvicK*, and Δ*ciaH* strains were cultured in medium with or without the addition of calcium ions. Bacterial cell wall-associated proteins were isolated by use of 8 M urea and were subjected to Western blot analysis using anti-AtlA antibodies. Calcium ion-enhanced AtlA maturation was abolished in the *vicK*-deficient strain. (B) eDNA released into the medium of *S. mutans* GS5, Δ*vicK*, com*ΔvicK*, and Δ*ciaH* strains cultured with or without 100 μM calcium ions was PCR amplified using bacterial 16S rRNA primers, and products were resolved on 1% agarose gels. (C) The biofilms of *S. mutans* GS5, Δ*vicK*, com*ΔvicK*, and Δ*ciaH* strains grown in medium with or without purified bacterial eDNA and/or 0.1 mM calcium ions were stained with 0.1% crystal violet and were quantified by measuring the absorbance at 550 nm. Data are means ± standard deviations from triplicate experiments. Asterisks indicate significance (***, $P < 0.001$) by one-way ANOVA. These experiments were repeated three times, and results of a representative experiment are shown. (D and E) The role of VicK in the pathogenesis of *S. mutans* in IE was further investigated using the Δ*vicK* and com*ΔvicK* strains in a rat model of IE. Vegetation size (D) and the numbers of colonized bacteria inside vegetations (E) were measured. Data represent means ± standard error of the means and were statistically analyzed using the Kruskal-Wallis test followed by Dunn's test (**, $P < 0.01$; *, $P < 0.05$).

In summary, our current data demonstrate the role of VicK in sensing calcium ions in the circulation and regulating AtlA maturation, which contributes to eDNA release and bacterial biofilm formation on damaged heart valves in a rat model of *S. mutans*-induced IE. Since AtlA may be present in other viridans group streptococci and a homolog has also been found in other Gram-positive microorganisms, the results obtained from the present study provide important information regarding the pathogenesis of other forms of pathogen-induced IE.

MATERIALS AND METHODS

Bacterial strains and plasmid. *S. mutans* GS5 and isogenic deletion mutant strains were grown and maintained in brain heart infusion broth (Difco Laboratories Inc., Detroit, MI, USA). The construction of the Δ*atlA* and comp*ΔatlA* strains is described in our previous study (25), and that of the Δ*vicK* and Δ*ciaH* strains is described below. GFP-tagged bacteria were generated by transformation with a shuttle plasmid (pPDGFPuv) containing the *GFPuv* sequence described in our previous study (25). For the selection of antibiotic-resistant colonies after transformation, the growth medium was supplemented with chloramphenicol (5 μg/ml) (for the Δ*atlA* and comp*ΔatlA* strains), kanamycin (500 μg/ml) (for the Δ*vicK*, Δ*ciaH*, com*ΔvicK*, and comp*ΔatlA* strains), or spectinomycin (500 μg/ml) (for pPDGFPuv and the com*ΔvicK* strain).

Constructions of the Δ*vicK*, Δ*ciaH*, and com*ΔvicK* strains. All references to genomic loci are based on the *S. mutans* UA159 genome database (https://www.ncbi.nlm.nih.gov/genome/). To construct the Δ*vicK* and Δ*ciaH* strains without the polar effect, these genes in *S. mutans* GS5 were disrupted by the insertion of a promoterless kanamycin cassette using a ligation-PCR mutagenesis strategy (36). Briefly, the promoterless kanamycin resistance gene fragment was isolated from the pALH124 plasmid by EcoRI digestion (37). Fragments of approximately 500 bp located before and/or after *vicK* and *ciaH* in the genome were PCR amplified using the forward and reverse primers listed in Table 1. PCR products were digested with EcoRI, ligated with the kanamycin resistance gene fragment, and transformed directly into *S. mutans* GS5. Correct allelic replacement in the derived isogenic mutant strains was confirmed by PCR amplification of the replacement region and reverse transcription-PCR (RT-PCR) analysis to verify the loss

TABLE 1 Primers for this study

Primer	Sequence (5′ to 3′)	Target
VicKBF	AAGCTCTTGCCGTCTTT	*vicK*
VicKBR_EcoRI	GGAATTCCGGTGGGACTGGTTTAGGA	*vicK*
VicKAF_EcoRI	GGAATTCCGCCAGTAATCAAAGAACG	*vicK*
VicKAR	GTCTTGAAATTGGTGCT	*vicK*
CiaHBF	AACGTGCTGAACTTAACG	*ciaH*
CiaHBR_EcoRI	GGAATTCCGATAAAGCCAGAACACGA	*ciaH*
CiaHAF_EcoRI	GGAATTCCGTATTAGCAGCCATCTGG	*ciaH*
CiaHAR	AACTGCTTGGCAAAGAGT	*ciaH*
VicKPF_BamHI	CGCGGATCCGGTAATGAGTGCCATAGT	*vic* promoter
VicKPR_EcoRI	CCGGAATTCAATTTTCTTCATTATGAATACC	*vic* promoter
VicKF_EcoRI	CCGGAATTCATGACTAATGTGTTTGAATCA	*vicK*
VicKR_EcoRI	CCGGA TTCTGTTTCTGTCATGATTCG	*vicK*

of *vicK* and *ciaH* expression and to confirm the expression of genes downstream of *vicK* and *ciaH*. For the construction of the com*ΔvicK* strain, gene fragments of the *vic* operon promoter and *vicK* were PCR amplified using the primers listed in Table 1. PCR products were digested with BamHI and EcoRI and were cloned into the *S. mutans–E. coli* shuttle vector, PDL278, at the BamHI and EcoRI sites (25). The resulting plasmid was transformed into the Δ*vicK* strain to generate the com*ΔvicK* strain. The expression of *vicK* was further confirmed by RT-PCR.

Biofilm formation assay. To establish sucrose-independent biofilms, sucrose-free defined M4 medium was used. Bacterial biofilm growth was initiated by inoculating individual wells of a 96-well polystyrene microtiter plate with approximately 10^7 CFU in 200 μl of M4 medium. To investigate the roles of bacterial eDNA and cell lysis in biofilm formation, DNase I (2 U/ml) and PAS (500 μg/ml) were added to the medium, respectively. After a 16-h incubation at 37°C, the medium was removed and the wells rinsed gently three times with sterile distilled water. The plates were then air dried, stained with 0.1% crystal violet for 10 min, rinsed three times with distilled water, and air dried again for 10 min. Each assay was performed in triplicate, and wells without biofilms were used as blank controls. Biofilms were quantified by measuring the absorbance at 550 nm using a MicroELISA reader (Dynatech Corp., Alexandria, VA, USA).

For CLSM analysis, *GFPuv*-tagged strains were transformed with pPDGFPuv, and the bacterial biofilms were cultured in a 24-well plate with a round glass coverslip in individual wells. After a 16-h incubation, biofilms that had formed on the glass coverslip were gently washed three times with phosphate-buffered saline and then fixed with 2% paraformaldehyde for 15 min. After fixation, bacterial eDNA embedded in the biofilm was stained with 10 μM PI. After three washes with phosphate-buffered saline, the coverslips were transferred to a slide and were observed by CLSM (Leica TCS SP5 confocal microscope).

Purification and semiquantification of eDNA. To detect eDNA release, the culture medium was collected and analyzed. Bacteria were cultured in M4 medium with or without PAS (500 μg/ml) and calcium ions. Before collection of the culture medium, biofilms adherent to the culture tubes were disrupted and suspended by violent agitation using a vortex. Bacterial cells were removed by centrifugation for 2 min at 16,000 $\times$ *g* andt 4°C. To specifically detect eDNA release, 1 μl of supernatant was used as a template for semiquantitative PCR (25 cycles) using specific bacterial 16S rRNA primers. M4 medium (1 μl) without seeding bacteria was used as a negative control, and medium containing *S. mutans* GS5 genomic DNA was used as a positive PCR control.

eDNA released into the culture medium was purified by collecting 0.5 ml of the culture medium supernatant and saturating it with a phenol-chloroform-isoamyl alcohol mix (25:24:1). After vortexing for 30 s, the mixture was centrifuged at 4°C for 5 min at 16,000 $\times$ *g*. The aqueous phase (0.3 ml) was removed and was mixed with 30 μl of 5 M NaCl and 800 μl of 100% ethanol. The mixture was first incubated at −80°C and then centrifuged for 10 min at 16,000 $\times$ *g*. The supernatant was decanted, and the precipitated sample was air dried and was suspended in 30 μl of double-distilled water. For the biofilm assay, 20 μl of purified eDNA was added to 200 μl of M4 medium. The total DNA of the vegetation was extracted using a Gentra Puregene tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The bacterial eDNA was detected by semiquantitative PCR (25 cycles) using specific bacterial 16S rRNA primers.

***S. mutans*-induced IE rat model.** Approval of animal use was obtained from the National Taiwan University Institutional Animal Care and Use Committee (Taipei, Taiwan, Republic of China) prior to initiation of the experiments. A modified rat model of experimental streptococcal endocarditis was used as described previously (38). For CLSM analysis, GFP-tagged wild-type *S. mutans* GS5 or its Δ*atlA* mutant (1×10^9 CFU) was injected into the tail veins of Wistar rats. The vegetation harvested from the heart of each rat was fixed on a glass slide and was stained with 10 μM PI. Bacterial biofilms were then observed under a confocal microscope (Leica TCS SP5).

Statistical analysis. The statistical significance of the difference between two sets of data was analyzed by using an unpaired, two-tailed Student *t* test. Differences between more than two sets of data were assessed using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple-comparison test. For nonparametrically distributed data, a Kruskal-Wallis test, followed by Dunn's test, was used. A *P* value of <0.05 was considered statistically significant.

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We declare that we have no conflicts of interest.

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