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Glycosyltransferase-Mediated Biofilm Matrix Dynamics and Virulence of *Streptococcus mutans*

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ABSTRACT *Streptococcus mutans* is a key cariogenic bacterium responsible for the initiation of tooth decay. Biofilm formation is a crucial virulence property. We discovered a putative glycosyltransferase, SMU_833, in *S. mutans* capable of modulating dynamic interactions between two key biofilm matrix components, glucan and extracellular DNA (eDNA). The deletion of *smu_833* decreases glucan and increases eDNA but maintains the overall biofilm biomass. The decrease in glucan is caused by a reduction in GtfB and GtfC, two key enzymes responsible for the synthesis of glucan. The increase in eDNA was accompanied by an elevated production of membrane vesicles, suggesting that SMU_833 modulates the release of eDNA via the membrane vesicles, thereby altering biofilm matrix constituents. Furthermore, glucan and eDNA were colocalized. The complete deletion of *gtfBC* from the *smu_833* mutant significantly reduced the biofilm biomass despite the elevated eDNA, suggesting the requirement of minimal glucans as a binding substrate for eDNA within the biofilm. Despite no changes in overall biofilm biomass, the mutant biofilm was altered in biofilm architecture and was less acidic *in vitro*. Concurrently, the mutant was less virulent in an *in vivo* rat model of dental caries, demonstrating that SMU_833 is a new virulence factor. Taken together, we conclude that SMU_833 is required for optimal biofilm development and virulence of *S. mutans* by modulating extracellular matrix components. Our study of SMU_833-modulated biofilm matrix dynamics uncovered a new target that can be used to develop potential therapeutics that prevent and treat dental caries.

IMPORTANCE Tooth decay, a costly and painful disease affecting the vast majority of people worldwide, is caused by the bacterium *Streptococcus mutans*. The bacteria utilize dietary sugars to build and strengthen biofilms, trapping acids onto the tooth's surface and causing demineralization and decay of teeth. As knowledge of our body's microbiomes increases, the need for developing therapeutics targeted to disease-causing bacteria has arisen. The significance of our research is in studying and identifying a novel therapeutic target, a dynamic biofilm matrix that is mediated by a new virulence factor and membrane vesicles. The study increases our understanding of *S. mutans* virulence and also offers a new opportunity to develop effective therapeutics targeting *S. mutans*. In addition, the mechanisms of membrane vesicle-mediated biofilm matrix dynamics are also applicable to other biofilm-driven infectious diseases.

KEYWORDS *Streptococcus mutans*, biofilms, eDNA, extracellular matrix, glucans, glycosyltransferases

Dental caries, commonly known as tooth decay or cavity, is characterized by the demineralization and subsequent destruction of tooth enamel, resulting in the formation of cavities. *Streptococcus mutans* is considered a major etiological agent because it can produce acids from the catabolism of various sugars that cause disso-

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lution of the tooth enamel, survive the low pH niche created largely by itself, and colonize and accumulate on the tooth surface in a robust acidic biofilm community (1, 2). The biofilm also protects the bacteria housed within it and traps acids close to the tooth surface, facilitating the development of carious lesions. The extracellular biofilm matrix of *S. mutans* consists of glucan polysaccharides, extracellular DNA (eDNA), and proteins. The most important and well-studied component of the biofilm is the glucan matrix, which provides a platform for *S. mutans* to adhere to the tooth surface, mechanical stability, and acidic microenvironments (3). The glucan matrix is synthesized and organized by extracellular glucosyltransferases (GtfB, GtfC, and GtfD). Under high-sucrose conditions, Gtfs break down sucrose and polymerize the glucose moiety into sticky glucose polymers. GtfB synthesizes water-insoluble glucans, GtfD synthesizes water-soluble glucans, and GtfC synthesizes a mixture of both (4, 5). Deletion of GtfB has been shown to be sufficient to abolish microcolony formation within the biofilm, and deletion of both GtfB and GtfC results in a severe defect in *S. mutans* biofilm formation (6) and bacterial cariogenicity (7).

Extracellular DNA (eDNA) is a major component of the biofilm extracellular matrix of many bacterial species. eDNA plays an important role in bacterial adhesion and accumulation, as well as the architecture and stability of biofilms (8–11). Additionally, eDNA is crucial for the formation of dental plaque biofilms, especially in the early stages (12). Programmed cell death and lysis are considered the major source of eDNA (13, 14); however, there is also growing evidence that DNA can be actively released via the type IV secretion system, membrane vesicles, and probably other lysis-independent mechanisms (11, 15–19). In *S. mutans*, it has been shown that DNA release can occur through cell death induced by the competence-stimulating peptides CSP/XIP (20, 21) and via secreted membrane vesicles in a lysis-independent manner (11). eDNA has been shown to be important in early *S. mutans* biofilm development, as pretreatment of *S. mutans* biofilms with DNase reduces the biofilm formation (11, 22). In addition, recent studies have suggested that in the presence of glucans, eDNA can further enhance *S. mutans* adherence (11). However, little is known regarding how various matrix components interact and contribute to the cariogenic biofilm formation. Understanding this new activity may reveal new targets that are amenable to the design of selective antibiofilm inhibitors.

The genome of *S. mutans* UA159 encodes several uncharacterized glycosyltransferases, enzymes responsible for the transfer of a sugar moiety from an activated nucleotide-sugar to an acceptor, which may be a lipid, protein, or growing oligosaccharide. Glycosyltransferases in *S. mutans*, including the aforementioned and well-characterized glucosyltransferases (GtfB, GtfC, and GtfD), have been shown to be vitally important for the organism's fitness and virulence and play important roles in cell wall biogenesis, biofilm formation, and protein homeostasis (4–6, 23–26). Our lab has been interested in uncovering the role of the putative glycosyltransferases with no known function. The *smu_833* gene is predicted to encode a hypothetical glycosyltransferase highly conserved among mutans group streptococci but absent among oral commensals. The SMU_833 protein is predicted to have two transmembrane helices and shares high homology with proteins involved in cell wall biogenesis and cell stress response, suggesting this protein may be important for the establishment, survival, or virulence of *S. mutans*.

In this study, we further investigated the function of SMU_833 and showed that the loss of SMU_833 resulted in a significant decrease in amounts of Gtfs and a concurrent reduction in glucan matrix but without any major effect on total biofilm biomass. Further analysis revealed an increase in extracellular DNA and an increased production of membrane vesicles. It appears that the increased eDNA and membrane vesicle production were able to compensate the loss of glucans to maintain the biofilm biomass but were not sufficient to replace the loss of glucans to generate a cariogenic biofilm. Despite no apparent loss of *in vitro* biofilm biomass, the lack of SMU_833 led to defects in bacterial colonization and caries formation in a rat caries model, demonstrating that SMU_833 is an important *S. mutans* virulence factor.

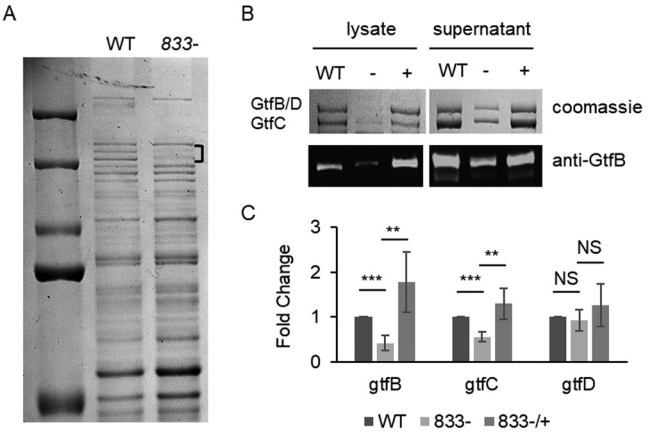

FIG 1 Effect of *smu_833* deletion on protein expression. (A) Coomassie-stained SDS-PAGE gel comparing total protein profiles of the *smu_833* mutant and its parent strain, UA159. Bracket indicates bands with decreased expression. (B) SDS-PAGE and Western blot of Gtf proteins in both the whole-cell lysate and cell supernatant from wild-type (WT), *smu_833* mutant (−), and complementation (+) strains. A GtfB-specific antibody was used in the Western blot. (C) qRT-PCR analysis of *gtfB*, *gtfC*, and *gtfD* gene expression levels in WT, *smu_833* mutant, and complement strains. Data presented here represent the mean values from four independent experiments. ***, $P < 0.001$; **, $P < 0.01$; NS, not significant compared with the wild type. Statistical analysis was done using Student's *t* test.

RESULTS

Deletion of *smu_833* affects production of high-molecular-weight proteins. SMU_833 is a putative glycosyltransferase whose function has yet to be elucidated. To gain insight into the function of SMU_833 in *S. mutans*, an in-frame deletion mutant for *smu_833* was constructed, confirmed, and characterized. The protein profiles of cell lysates from the parent strain UA159 and the *smu_833* deletion mutant were examined by SDS-PAGE analysis. Although the banding patterns of most proteins were similar between the wild-type strain and the mutant, there were reduced amounts of two protein bands just above 150 kDa in the mutant lysate (Fig. 1A). Mass spectrometry analysis of the protein bands revealed they are GtfB, GtfC, and GtfD (data not shown). These proteins are known to be secreted and are needed for the formation of the biofilm glucan matrix. Because the majority of these proteins are found in the culture supernatants (4), levels of Gtfs were further analyzed in both the cell lysates and cell supernatant fractions (Fig. 1B, top panel). A decrease in Gtfs in both fractions of the mutant was evident. In addition, Western blot analysis confirmed the decrease in GtfB in the *smu_833* mutant (Fig. 1B, bottom panel). Furthermore, complementation restored the amount of Gtfs in the mutant to the level found in the parent strain. Together, these data demonstrate that deletion of *smu_833* decreases overall Gtf levels and that the decrease is not due to the redistribution of protein localization.

Next, we determined whether the decrease in Gtf protein levels in the mutant was related to a decrease in transcript levels of the *gtf* genes. Reverse transcription-quantitative PCR (qRT-PCR) analysis revealed that the loss of SMU_833 resulted in a decrease in the transcript levels of *gtfB* and *gtfC* but not *gtfD* (Fig. 1C). This result suggests that SMU_833 affects the transcription of *gtfB* and *gtfC* and that the decrease in protein levels of the Gtfs is at least partially due to the decrease in transcript levels. GtfB and GtfD are not able to be separated by SDS-PAGE and, therefore, run as one band (Fig. 1B, the top band). It is possible that the decrease in the protein band for GtfB and GtfD is just a decrease in the levels of GtfB and the protein levels of GtfD have remained unchanged.

Loss of SMU_833 results in a large decrease in glucans within the bacterial biofilm but retains comparable total biofilm biomass. GtfB and GtfC are key enzymes needed to produce the glucan matrix component of the *S. mutans* biofilm. Because there was a decrease in the Gtfs in the *smu_833* deletion mutant, we hypothesized the *smu_833* mutant would yield less glucan matrix and have a reduced total

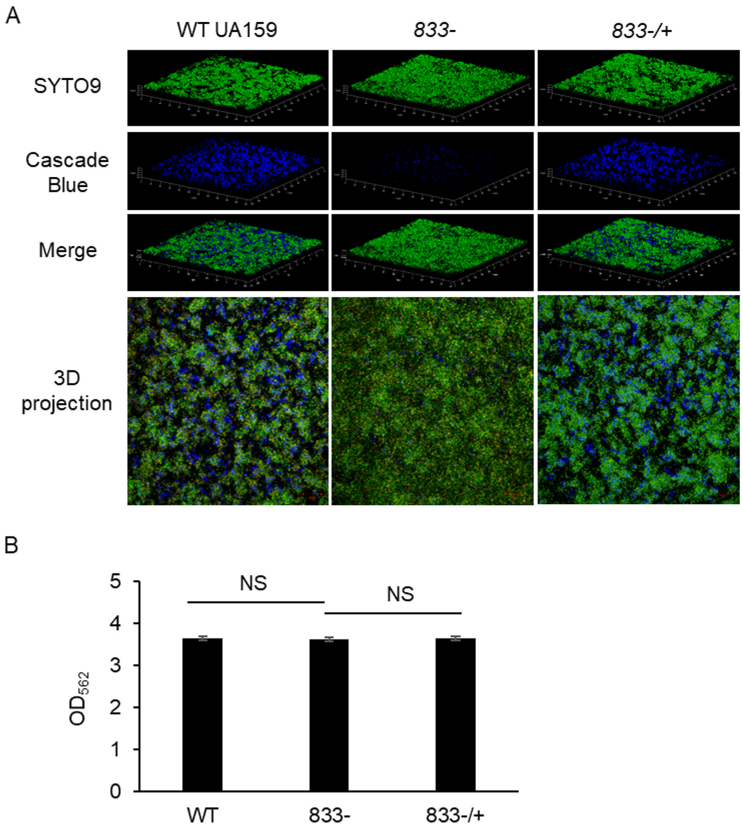

FIG 2 Visualization of bacterial biofilms and measurement of total biofilm biomass. (A) Confocal laser scanning microscopy of wild-type, mutant, and complement biofilms. Bacterial cells were stained with SYTO9 green, and the glucan matrix was stained with Cascade blue conjugated to dextran. Images are representative of at least three independent experiments. (B) Comparison of total biofilm biomasses produced by wild-type, *smu_833* mutant, and complementation strains. Biofilms were grown on 96-well polystyrene plates in TYE medium supplemented with 1% sucrose for 18 h. Biofilms were stained with crystal violet, and OD_{562} was read to ascertain total biofilm biomass. Data are representative of those from at least three independent experiments for which there was no significant difference (NS; $P > 0.05$). Statistical analysis was done using Student's *t* test.

biofilm biomass. To determine whether a decrease in Gtfs translated to the production of less glucan matrix, we used confocal laser scanning microscopy (CLSM) to evaluate the *S. mutans* biofilm. The parent strain formed distinct microcolonies with an abundant glucan matrix to support the biofilm structure (Fig. 2A, left panel). However, the mutant biofilm had greatly reduced glucan matrix formation and showed an altered structure with less-defined microcolonies (Fig. 2A, middle panel). The biofilm formed by the complement strain displays a phenotype similar to that of the parent strain (Fig. 2A, right panel). Despite the large decrease in the glucan matrix, the mutant appears to yield a large biofilm, comparable in overall mass with the wild type (Fig. 2A, top panel; SYTO9). To quantitatively determine if the total biofilm biomass was altered by the loss of *smu_833*, crystal violet staining of biofilms was performed. Surprisingly, despite the decrease in the major structural component of the biofilm, the *smu_833* mutant was still capable of producing a total biomass that was comparable to the parent strain (Fig. 2B), suggesting other components of the *S. mutans* biofilm matrix may be increased to compensate for the glucan defect. Nevertheless, these data illustrate that SMU_833 is important for the glucan matrix formation and the biofilm structure.

Loss of SMU_833 increases extracellular DNA but does not significantly change cell viability. Extracellular DNA (eDNA) is another component of the *S. mutans* biofilm which is known to play an imperative role in the biofilm formation of many other bacterial species, as well as early biofilm formation in *S. mutans* (11, 22, 27). To determine if the *smu_833* mutant produces more eDNA than its parent strain, super-

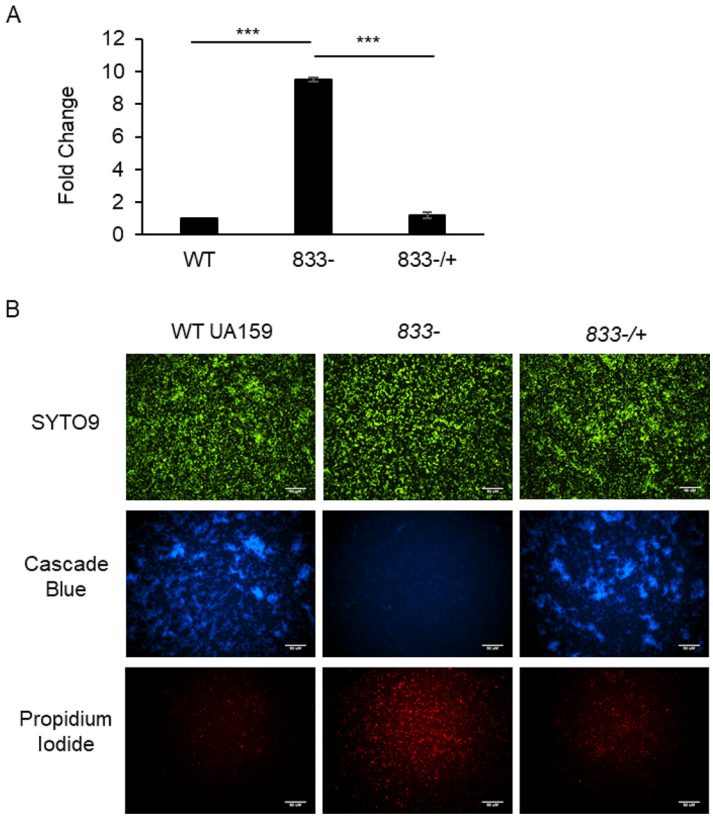

FIG 3 Measurement and visualization of extracellular DNA in planktonic cultures and biofilm. Comparison of extracellular (eDNA) levels in wild-type, *smu_833* deletion mutant, and complemented bacterial strains. (A) Real-time PCR using 16S rRNA specific primers was used to analyze the eDNA concentration in the supernatants of planktonic cultures grown to an OD_{470} of 1.0. Data presented here represent the average of values from three independent experiments. ***, $P < 0.001$. Statistical analysis was done using Student's *t* test. (B) Fluorescence microscopy was used to visually determine the relative amount of eDNA and cell death within the bacterial biofilms grown for 5 h. The biofilm glucan matrix was stained using Cascade blue conjugated to dextran, SYTO9 green for DNA of living cells, and propidium iodide red for DNA in dead cells/eDNA within the biofilm. Images are representative of at least three independent experiments.

natants of planktonic bacterial cultures were first analyzed for relative eDNA concentration via quantitative PCR (qPCR) using primers specific to *S. mutans* 16S rRNA. The supernatant of the mutant contained almost 10-fold more eDNA than that of UA159 (Fig. 3A). The DNA levels in the complementation strain were similar to those in the wild type, indicating that the increase in eDNA in the *smu_833* mutant was due to the deletion of *smu_833* (Fig. 3A). To determine if the *smu_833* deletion also caused an increase in eDNA within the biofilm, levels of eDNA were visualized with fluorescence microscopy. Early biofilms (5 h) were used for easier visualization of eDNA and to avoid the use of mature biofilms, which often contain too many dead cells. The biofilm of wild-type (WT) UA159 contained a large amount of glucan matrix but only a small amount of eDNA/dead cells (Fig. 3B, left panel). In contrast, the *smu_833* mutant biofilm contained very little glucan matrix but a larger quantity of eDNA/dead cells than the WT UA159 (Fig. 3B, middle panel). Complementation of *smu_833* again restored levels of glucan matrix and eDNA/dead cells to wild-type levels (Fig. 3B, right panel). Again, the overall biomass produced by each strain appeared equal to the others (Fig. 3B, top panel; SYTO9). Together, these results suggest that the eDNA levels increased within the *smu_833* mutant and that the increase in eDNA helps to compensate for the lack of glucan matrix.

One possible reason for an increase in eDNA is an increase in cell death and lysis. To determine if deletion of *smu_833* reduces cell viability, cultures from wild-type UA159,

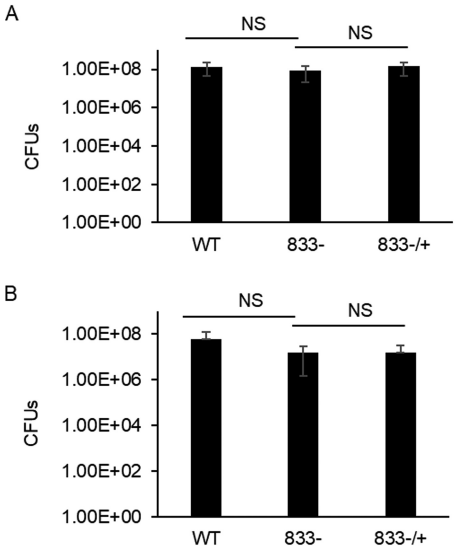

FIG 4 Viability of wild-type UA159, *smu_833* mutant, and complement strains in planktonic and biofilm cultures. Bacterial cultures were either grown planktonically in THB to an OD_{470} of 1.0 (A) or allowed to form a biofilm for 5 h in TYE with 1% sucrose (B). Appropriate dilutions of the cultures were made and plated onto THB plates, and CFU were enumerated. Data are averages of those from at least five independent experiments. NS, no significant difference ($P > 0.05$). Statistical analysis was done using Student's *t* test.

the *smu_833* mutant, and its complement were grown to exponential phase, and their viability was measured by counting colony forming units (CFU) (Fig. 4A). Exponential-phase cultures were used to match the time point used in Fig. 3. There was no significant difference in the cell viability between the strains, indicating that the large increase in eDNA in the mutant is not due to an increase in cell lysis. A similar assay was performed to determine if the loss of *smu_833* caused an increase in cell lysis within the bacterial biofilm. Again, no significant change in cell viability was observed with the deletion of *smu_833* (Fig. 4B). To corroborate these data, alamarBlue was used as another means to assess cell viability within the biofilm. alamarBlue staining also indicated that there is no significant difference in cell viability between these strains (Fig. S1). Taken together, these results indicate that *smu_833* deletion does not cause a discernible decrease in cell viability. It is likely that a cell lysis-independent mechanism accounts for the increase in eDNA.

Deletion of *smu_833* results in an increase in production of membrane vesicles and eDNA. Membrane vesicles are known to be a source of eDNA in Gram-negative bacteria and similar results have also been recently reported in Gram-positive bacteria (15–17). *S. mutans* has been shown to be capable of producing DNA containing membrane vesicles in a lysis-independent manner (11). To determine if the *smu_833* deletion mutant produces more membrane vesicles, membrane vesicles were prepared from cell-free supernatants by ultracentrifugation and estimated using the bicinchoninic acid (BCA) protein assay. The results showed that the mutant contained 2.54-fold more membrane vesicle proteins, indicative of an increase in vesicle production (Fig. 5A). In addition, bacterial DNA concentrations of vesicle preparations were measured by real-time quantitative PCR. The SMU_833 membrane vesicles contained at least 50-fold-higher concentrations of DNA than the wild-type and complemented strains (Fig. 5B). These data support the notion that eDNA released by membrane vesicles in the *smu_833* mutant contributes to the biofilm matrix.

Glucan interacts with eDNA and is required to form a biofilm. Glucans are an important component of the *S. mutans* biofilm matrix. We have found that the SMU_833-deficient mutant is capable of forming a biofilm with a biomass similar to that of the wild type but with a greatly reduced glucan matrix. However, it remains to be

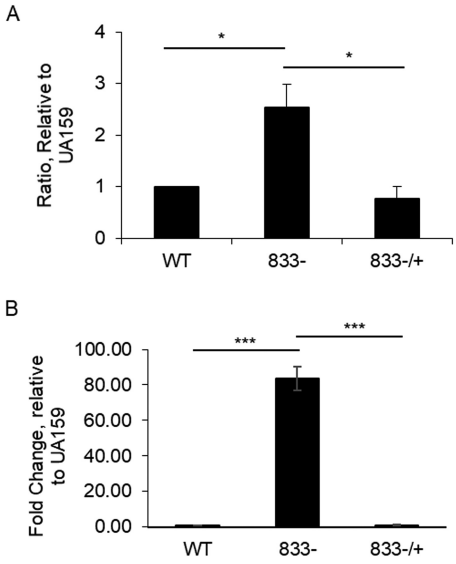

FIG 5 Analysis of membrane vesicle production and DNA content. Membrane vesicle yield was estimated by measuring protein concentration using BCA assay. (A) Ratio of membrane vesicle production relative to *S. mutans* UA159. Data represent the average (±SD in error bars) values from three independent sets of experiments. *, $P < 0.05$ versus UA159. (B) DNA content of membrane vesicles was measured with real-time PCR using 16S specific primers. Results presented are representative of those from at least three independent experiments. ***, $P < 0.001$. Statistical analysis was done using Student's *t* test.

determined if this mutant can form a biofilm in the complete absence of Gtfs. To determine this, each *gtf* gene was deleted from the *smu_833* mutant, and the total biofilm biomasses of different deletion variant strains were compared. A deletion of *gtfC* did not affect the total biomass of the biofilm formed in a rich medium, tryptone-yeast extract (TYE) (Fig. 6A). However, a deletion of *gtfB* caused a large reduction in biofilm formation. The double deletion of *gtfB* and *smu_833* did not further reduce biofilm formation compared with that of the *gtfB* mutant (Fig. 6A), while double deletion of *gtfB* and *gtfC* resulted in an even greater reduction. In addition, the triple deletion of *gtfB*, *gtfC*, and *smu_833* led to the greatest reduction in the biofilm biomass (Fig. 6A). Complementation of *smu_833* in both the *gtfB smu_833* double mutant and the *gtfBC smu_833* triple mutant increased biofilm formation beyond the levels of either the double or triple mutant. These results suggest that despite the increase in eDNA, a certain amount of glucan matrix is still required for the *smu_833* mutant to support biofilm formation.

Because our data suggest that some glucans are needed for the *smu_833* mutant to form a biofilm despite an increase in eDNA, we hypothesize that glucan may prime the binding of eDNA, and, thus, the two components directly interact within the *S. mutans* biofilm. In a cell-free matrix formation system, Gtf proteins and eDNA prepared from the supernatant of wild-type *S. mutans* were allowed to form glucans in TYE medium supplemented with 1% sucrose. This cell-free system was used to simplify and better visualize possible interactions between matrix components. Fluorescence microscopy revealed that eDNA and glucan colocalized with one another (Fig. 6B). This interaction and the need for the presence of some glucans together suggest that glucans and eDNA are integral components of the biofilm matrix.

Loss of SMU_833 results in an increased biofilm pH. As the *S. mutans* biofilm develops, more bacteria accumulate and ferment more sugars, which cause the pH of the biofilm to drop and demineralization of tooth enamel to occur. The biofilm matrix facilitates the formation of punctate areas of low pH next to the surface on which the biofilm is attached (28). Because the matrix and the overall structure of the mutant biofilm differ from those of wild-type UA159, we evaluated the pH of the biofilms. In the wild-type biofilm (Fig. 7, left panel), there were many areas with an intense red coloring signifying many areas of low pH. However, in the mutant biofilm (Fig. 7, middle panel),

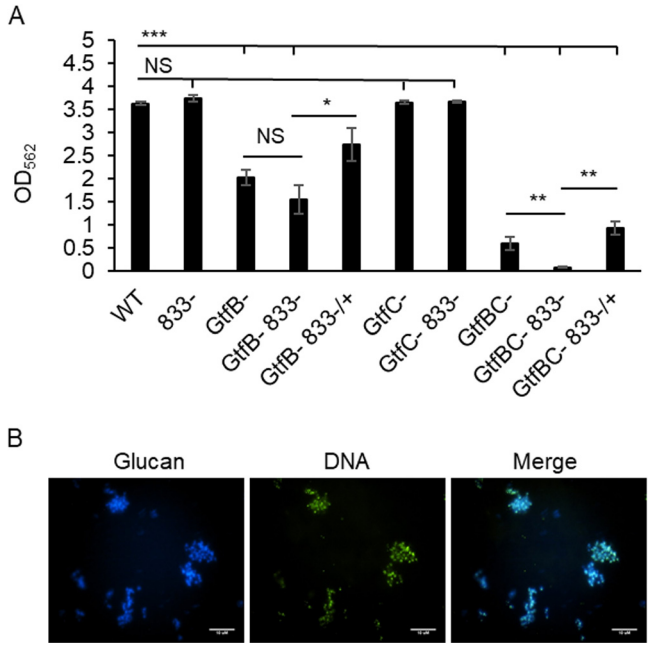

FIG 6 Glucan contribution to biofilm formation. (A) Strains containing a Gtf deletion with and without a deletion of *smu_833* were allowed to form a biofilm for 18 h in TYE medium supplemented with 1% sucrose. Biomass was quantified with crystal violet staining, and the optical density at 562 nm was read. Results are representative of those from at least three independent experiments. NS, $P > 0.05$; *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$. Statistical analysis was done using Student's *t* test. (B) Filtered culture supernatants containing eDNA and Gtf proteins were incubated with sucrose overnight and allowed to form cell-free biofilms, which were visualized with fluorescence microscopy. Cascade blue conjugated to dextran was used to visualize glucan, and SYTO9 green was used for visualization of eDNA.

the majority of the fluorescence was of a low intensity, indicative of a less acidic pH. The complemented strain regained wild-type levels of red fluorescence intensity and area coverage (Fig. 7, right panel). These results indicate that the *smu_833* deletion leads to the formation of a less acidic biofilm.

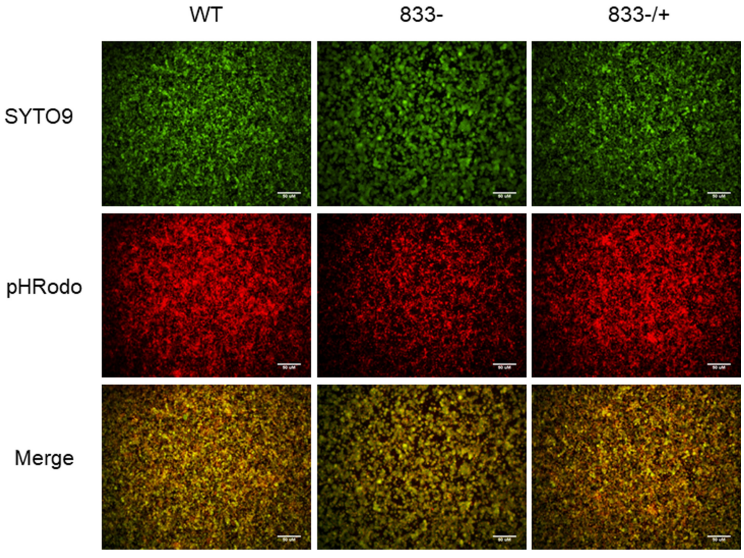

FIG 7 Effect of *smu_833* deletion on biofilm pH. Wild-type, *smu_833* mutant, and complemented bacterial strains were allowed to form a biofilm on polystyrene plates for 16 h. pHRodo red conjugated to dextran was added after the formation of the biofilm. An increase in red is indicative of a decrease in pH. SYTO9 green was used to stain bacterial cells. Images are representative of those from three independent experiments.

TABLE 1 Colonization and cariogenicity of *S. mutans* wild type and *smu_833* mutant in rats[a]

Group	Mean ± SEM caries score by location and severity[b]										*S. mutans* (CFU/ml [× 10^6])[c]
	Buccal				Sulcal				Proximal		
	E	DS	DM	DX	E	DS	DM	DX	E	DS	
UA159	13.0 ± 0.7	9.4 ± 0.7	7.4 ± 0.5	5.0 ± 0.5	20.2 ± 0.9	15.0 ± 0.3	8.6 ± 0.5	4.4 ± 0.4	5.2 ± 0.8	2.8 ± 0.5	11.0 ± 0.3
smu_833 mutant	8.8 ± 0.7	6.2 ± 0.4	4.4 ± 0.6	1.8 ± 0.4	16.2 ± 0.4	11.2 ± 0.2	4.8 ± 0.6	1.2 ± 0.5	2.8 ± 0.8	0 ± 0	4.3 ± 0.6
Statistical significance	**	**	**	***	**	***	***	***	NS	***	***

[a]$n = 5$ per group. Weights (g): wild type, 136 ± 11; *smu_833* mutant, 128 ± 10. There was no significant difference between the weights of the groups. **, $P < 0.01$; ***, $P < 0.001$; NS, no significance.
[b]E, enamel; DS, dentinal slight; DM, dentinal moderate; DX, dentinal extensive.
[c]Values were determined by plating plaque samples on mitis salivarius (MS) agar.

SMU_833 mutant exhibits reduced bacterial colonization and cariogenicity *in vivo*. The *smu_833* mutant forms a biofilm that exhibits an alternate structure and a higher pH environment, which may become less cariogenic. An established rat caries model (29–33) was used to determine whether the loss of *smu_833* had any impact on *S. mutans* colonization and cariogenicity *in vivo*. Rats were infected with either the wild-type *S. mutans* or the *smu_833* mutant and fed a 5% sucrose diet to aid in the formation of *S. mutans* biofilms. The results showed that there were significantly fewer carious lesions in rats infected with the mutant (Table 1). Additionally, mutant-infected rats yielded fewer recoverable *S. mutans* CFU (Table 1). Taken together, these data indicate that SMU_833 is a virulence factor that contributes to bacterial colonization and cariogenicity.

DISCUSSION

The biofilm matrix produced by cariogenic bacteria is the scaffold that shapes and strengthens the biofilm, and it enables the formation of acidic microenvironments, which facilitate demineralization of tooth enamel and caries formation (34). One of the most important components of the biofilm matrix is the glucan polymers produced by the glucosyltransferases GtfB and GtfC secreted by *S. mutans*. In this study, we characterized a putative glycosyltransferase, SMU_833, and determined that deletion of *smu_833* resulted in an unexpected decrease in GtfB and GtfC levels and, subsequently, reduced glucan production within the biofilm. A point mutation of the catalytic DXD motif of SMU_833 results in the same reduced Gtf and glucan phenotype (Fig. S2). Additional studies are required to determine the exact mechanism by which SMU_833 causes a reduction in Gtfs. Protein glycosyltransferase of streptococci (PgfS) modifies and prevents degradation of the collagen binding protein Cnm in *S. mutans* (25). As SMU_833 is a putative glycosyltransferase, it is possible that SMU_833 functions in a similar manner and is needed for glycosylation and stability of the Gtfs.

Gtfs are the enzymes responsible for the building of the sticky glucan matrix, which allows *S. mutans* to adhere rigorously to the tooth surface (4, 23, 35). Therefore, it was not surprising to observe a decrease in glucan matrix production in the *smu_833* mutant biofilm. Interestingly, the mutant still maintained a total biofilm biomass comparable with that of the parent strain. This result was unexpected, as it has been well documented that the glucan matrix is key to the formation of robust *S. mutans* biofilms and that the loss of Gtfs often results in little to no biofilm being formed (6, 23, 35). This finding may reveal a new dynamic in the biofilm matrix. Indeed, we discovered that the amount of extracellular DNA was elevated in the *smu_833* mutant, which likely compensated for the loss of the glucan matrix. It is unclear how *S. mutans* may be able to coordinate this dynamic modulation. It has been shown that the exopolysaccharide Pel can compensate for the lack of a different exopolysaccharide, Psl, in *Pseudomonas aeruginosa* (36, 37). However, the underlying mechanism for this remains unknown. It was speculated that the presence of the Psl polysaccharide can act as an environmental signal that downregulates Pel production (36). In a similar manner, *S. mutans* may be able to sense the reduction in glucans and, in response, upregulate DNA release. In

addition, it was shown that polysaccharide biofilm matrix components can be upregulated in response to the loss of surface adhesin proteins LapA and LapF in *Pseudomonas putida*, and it was suggested that the second messenger molecule cyclic di-GMP may play a role in the balance of matrix components (38). In *S. mutans*, altered levels of the second messenger c-di-AMP can affect biofilm formation (32, 39), which may be a possible means by which the *smu_833* mutant can alter its biofilm.

eDNA plays an important role in biofilm formation in many bacterial species and has been shown to play a role in early biofilm formation in *S. mutans* (11, 22, 27). Both cell lysis-dependent and -independent mechanisms of DNA release have been proposed in *S. mutans* (11, 20, 21). Our data suggest that the increased eDNA present in the *smu_833* mutant is a result of the active release of membrane vesicles and not due to an increase in cell lysis. Emerging studies provide evidence of the presence of eDNA and increased secretion of nucleic acid-containing membrane vesicles in Gram-positive bacteria, including *S. mutans* (11, 16, 17, 40); however, how they contribute to the biofilm matrix or how they are regulated is not defined. It is possible that the cargo for the membrane vesicles packaged in *S. mutans* is linked to a regulatory network by which the bacteria may be able to sense the decrease in glucans and, thereby, upregulate eDNA release into the biofilm via vesicles. Furthermore, a decrease in the production of glucans led to an increase in pH. Acidic pH can become a signal sensed by some bacteria, including *S. mutans*, to induce the gene expression controlled by bacterial two-component regulatory systems (41, 42). Changes in this gene expression pattern may lead to the activated secretion of outer membrane vesicles and eDNA. Further investigation into the regulation of alternate matrix components would provide new insights into our understanding of *S. mutans* matrix biology.

A recent study of *S. mutans* biofilms using field emission scanning electron microscopy analysis indicates interactions between nanofibrous eDNA and wool-like glucans (11), similar to what has been reported in *Myxococcus* spp. (8). The results from our current study demonstrate the dependence of the biofilm formation on the interaction between glucan matrix and eDNA. Elevated eDNA can compensate for the partial loss of glucans but not the complete loss of glucans. The biofilm formation supported by increased eDNA only occurs when small amounts of glucans exist. Furthermore, the glucan and eDNA of *S. mutans* colocalize, suggesting that the glucan acts as a scaffold for eDNA to bind. This further demonstrates the importance of glucans in biofilm formation and reveals the dynamic interactions between two matrix components. It has been reported that eDNA and glucan matrix components of biofilms interact with one another in *P. aeruginosa* (43) and the negative charge of eDNA allows for binding to occur with the positively charged polysaccharides of *P. aeruginosa* (36). Furthermore, eDNA can acidify the biofilm and trigger the formation of acidic biofilms in *P. aeruginosa* (41). This is different from *S. mutans* where, despite an increase in eDNA, the net pH increases upon the deletion of *smu_833* and the reduction of Gtfs. In *S. aureus*, eDNA was shown to facilitate the formation of functional amyloids in biofilms (44). *S. mutans* is also shown to form amyloids, structured protein fibrils which, too, contribute to biofilm formation (45). However, the exact mechanism for how glucans, eDNA, and amyloids of the major biofilm matrices interact in *S. mutans* is unclear and needs further investigation. The DNABII family of DNA-binding proteins have also been shown to play a role in the structure and stability of DNA within biofilms (46–49), and it is possible that proteins such as these also play a role in the matrix interactions in *S. mutans*.

Despite the formation of a comparable *in vitro* biofilm biomass by the *smu_833* mutant, the biofilm structure and composition were altered, resulting in a higher biofilm pH suggestive of a reduced cariogenic potential. Our studies revealed that SMU_833 is an important virulence factor *in vivo*. The *smu_833* mutant reduced bacterial colonization and attenuated bacterial virulence in a rat model of dental caries. Because both the wild-type and mutant strains were able to colonize the rat initially, reduced colonization of the mutant at the end of the experiment suggests either the altered matrix was unable to facilitate bacterial adherence to the tooth surface as well or the bacteria themselves were unable to compete with the other bacteria present in

the oral cavity. Further studies are ongoing to determine the fitness and competitiveness of the *smu_833* mutant. The complex biofilm architecture supported by the glucan matrix is required for the creation of acidic, cariogenic microenvironments (28), which is essential for bacterial virulence. Our findings reveal a new virulence factor, SMU_833, whose deficiency alters the dynamic of the glucan and eDNA matrices of *S. mutans* biofilm and, thus, modulates bacterial virulence. It is still unclear precisely how SMU_833 affects the production of Gtfs and the release of eDNA by membrane vesicles.

In *Enterococcus faecalis*, eDNA is localized at the septum and the release may occur from metabolically active, living cells with a membrane potential (15). This may be the mechanism responsible for the active secretion of eDNA by membrane vesicles reported for *S. mutans*. In this regard, the *smu_833* gene is located downstream of and adjacent to the gene cluster (*smu_825* to *smu_831*) that encodes the biosynthetic pathway for the rhamnose-glucose polysaccharides (RGPs) located on the surface of *S. mutans* (24, 50). However, neither the mutant unable to produce any surface RGPs (Δ*rgpG*) nor the mutant that produces the rhamnose backbone without the glucose side chains (Δ*rgpE*) showed any decrease in glucan matrix (Fig. S3). This result indicates that the *smu_833* biofilm phenotype is independent of the RGPs. However, further studies are ongoing to determine if SMU_833 affects cell envelope biogenesis and membrane vesicle release by another means. Figure S4 shows that the *smu_833* mutant is more susceptible to autolysis by Triton X. Therefore, although the mutant is viable and metabolically active, the deletion of *smu_833* may affect the cell wall or membrane in some manner, decreasing its stability under certain stressors. This could be a potential explanation for an increase in membrane vesicles or possible evidence that an alteration in the cell wall or membrane has made the bacterial cells more "leaky" and able to release DNA or other cell materials more readily. More studies are underway to further elucidate the role of SMU_833 in *S. mutans*. Understanding the underlying mechanisms may reveal new targets that can be used to develop potential therapeutics that prevent and treat dental caries.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptococcus mutans* UA159 (51) and *Escherichia coli* Top 10 (Invitrogen) were used in this study. *S. mutans* strains were maintained in either Todd-Hewitt broth (THB) or tryptone-yeast extract (TYE) medium and grown statically at 37°C with 5% CO_2. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates and grown aerobically at 37°C. Antibiotics were used at the following concentrations: 1 mg ml^{-1} kanamycin and 10 μg ml^{-1} erythromycin for *S. mutans*, and 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} ampicillin, and 300 μg ml^{-1} erythromycin for *E. coli*.

Construction and complementation of mutants. Mutant strains were derived from *S. mutans* UA159 using the allelic replacement mutagenesis strategy by inserting a nonpolar kanamycin resistance cassette in place of *smu_833*. The *smu_833* open reading frame plus its 1-kb flanking regions was amplified from *S. mutans* UA159 genomic DNA using PCR. The fragment was ligated into a pGEM-T plasmid, transformed into *E. coli* Top10, and selected for using plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The plasmid was isolated, and inverse PCR adding XbaI restriction sites was used to amplify everything except *smu_833*. The PCR product and the kanamycin cassette were digested with XbaI, ligated, and transformed into *E. coli* using ampicillin and kanamycin for selection. The resulting plasmid was transformed into *S. mutans* UA159 and selected for using kanamycin (1 mg ml^{-1}). The replacement of *smu_833* with a kanamycin resistance cassette was verified by PCR. The complete open reading frame of *smu_833* was amplified and cloned into the *E. coli-Streptococcus* shuttle vector pVPT (57) to generate the corresponding complementation plasmid pVPT-*smu_833*. The plasmid was then transformed into the *smu_833* mutant strain to generate a complemented strain. The *gtfB*, *gtfC* single mutant and *gtfBC* double mutant in which *gtf* was replaced with a kanamycin resistance cassette, *aphA3* (encoding an aminoglycoside phosphotransferase), are gifts from Robert Burne's laboratory, University of Florida, Gainesville, FL. The pGEM-T plasmid containing the kanamycin resistance cassette and the regions flanking *smu_833* which was used to delete *smu_833* from WT *S. mutans* (described above) was used to create the *smu_833 gtfB* and *smu_833 gtfC* double mutants and the *smu_833 gtfB gtfC* triple mutant. Mutants were selected for using kanamycin (1 mg ml^{-1}) and verified with PCR.

SDS-PAGE and Western blotting. The same cell numbers of each bacterial sample were spun down and resuspended in lysis buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, and 0.5% [vol/vol] NP-40). Glass beads were added, and the samples were vortexed for 60 min. Cell debris was separated from the protein extracts by centrifugation at 9,279 × *g* for 10 min. Cell supernatants were harvested and precipitated with 30% ethanol at −80°C for at least 1 h. Supernatant proteins were harvested by centrifugation at 15,682 × *g* for 15 min at 4°C. Protein samples were then dissolved into SDS loading buffer and boiled for 10 min. Proteins were separated by SDS-PAGE, blotted onto polyvinyl difluoride

TABLE 2 Primers used in this study

Primer[a]	Sequence (5′–3′)	Description
Smu_833 pGEM F	CTTCCTGAAAAGGGTTATCTATATG	*smu_833* deletion
Smu_833 pGEM R	ACCTAACGCAAAAGACAAGG	*smu_833* deletion
Smu_833 Xba1 Inv F	CGCGTCTAGAAGGCTATAGGCATTTATTTAGAA	*smu_833* deletion
Smu_833 Xba1 Inv R	CGCGTCTAGATTTTCCCCCTTGAAAATTAATAATTGG	*smu_833* deletion
Smu_833 Kpn1 F	CGCACGGGTACCATGAAAAAACTTTCAATAG	*smu_833* complement
Smu_833 BspH1 R	GGTCAGTCATGAGTCCTTTTTCCTTAAC	*smu_833* complement
16S rRNA F	CTTACCAGGTCTTGACATCCC	qRT-PCR
16S rRNA R	CCAACATCTCACGACACGAG	qRT-PCR
gtfB F	ACACTTTCGGGTGGCTTG	qRT-PCR
gtfB R	GCTTAGATGTCACTTCGGTTG	qRT-PCR
gtfC F	CAAAATGGTATTATGGCTGTCG	qRT-PCR
gtfC R	TGAGTCTCTATCAAAGTAACGCAG	qRT-PCR
gtfD F	CGGTATTCAGGTTATTGCG	qRT-PCR
gtfD R	CTCACCATAATCGTTGACACG	qRT-PCR

[a]F, forward; R, reverse.

membranes, and incubated with a GtfB-specific antibody followed by goat anti-rabbit IgG. Signals were detected using Li-COR Biosciences Odyssey infrared imaging system.

Biofilm formation assay. *S. mutans* biofilms were grown in TYE medium (2.5% tryptone, 1.5% yeast extract) containing 1% sucrose. Overnight cultures were subcultured into fresh THB, grown to an optical density at 470 nm (OD_{470}) of 0.6, diluted to an OD_{470} of 0.01, subsequently aliquoted in a 96-well microtiter plate, and grown at 5% CO_2 at 37°C under static conditions. 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 (52). Each assay was carried out with at least triplicate samples and replicated at least three times.

Confocal laser scanning microscopy analysis. Biofilms were formed as stated above in TYE medium with 1 μM dextran-conjugated Cascade blue (Molecular Probes, Invitrogen) to label exopolysaccharide (EPS) on 8-well ibiTreat slides (catalog number 80826; ibidi) under 5% CO_2 conditions at 37°C for 16 h. The biofilm samples were gently washed with phosphate-buffered saline (PBS) three times to remove any nonadherent cells and then stained with SYTO9 green (Molecular Probes, Invitrogen). The stained samples were then examined by CLSM (Zeiss LSM 710 laser confocal microscope) with a 63× oil immersion objective. Images were obtained from serial optical sections. At least three independent experiments were performed on different days, and the images displayed are representative.

Biofilm eDNA fluorescence. Biofilms were formed as stated above in TYE medium supplemented with 1% sucrose with the addition of Cascade blue conjugated to dextran. Diluted cultures were plated onto a 24-well plate, and biofilms were grown statically for 5 h at 37°C in 5% CO_2. Biofilms were washed 3 times with PBS, and then SYTO9 and propidium iodide diluted in PBS were added to the wells. Fluorescence microscopy was performed using the 10× objective magnification on the Nikon Eclipse TE2000-E inverted fluorescence microscope. At least three independent experiments were performed on different days, and the images displayed are representative.

Visualization of biofilm pH with fluorescence microscopy. Biofilms were grown as stated above. After incubating for 16 h in 24-well plates, 1 μM dextran-conjugated pHRodo red (Molecular Probes, Invitrogen) was added to the wells and the mixture was incubated for 3 h at room temperature. The biofilms were then washed with PBS three times to remove any nonadherent cells and stained with SYTO9 (Molecular Probes, Invitrogen) diluted in PBS. Fluorescence microscopy was performed using the 10× objective magnification on the Nikon Eclipse TE2000-E inverted fluorescence microscope. At least three independent experiments were performed on different days, and the images displayed are representative.

Planktonic eDNA quantification. Overnight cultures were diluted into fresh THB and grown at 37°C in 5% CO_2 to an OD_{470} of 1.0. Cultures were centrifuged, and the supernatant was filtered with a 0.22-μm filter (Millex-GS). Quantitative PCR was performed using SYBR green master mix on an iCycler iQ5 machine (Bio-Rad). The *S. mutans* 16S rRNA gene was used to quantify the amount of DNA present in the supernatant and the delta *CT* method was used to calculate fold change.

Preparation of membrane vesicles and DNA measurement. Membrane vesicles (MVs) were prepared by following the protocol of Rivera et al. (11, 53, 54). *S. mutans* strains were grown in FMC medium with glucose (55) overnight (16 h) or in THB to an OD_{470} of 1.0 for DNA quantification. Following centrifugation at 6,000 × *g* at 4°C for 10 min, the cell-free culture supernatants were filtered through 0.22-μm syringe filters to remove residual cells. MVs in the cell-free supernatants were collected and washed once with sterile PBS after centrifugation at 140,153 × *g* at 4°C for 2 h. After being washed, the pellets were resuspended in 200 μl PBS. MV yield was estimated by measuring protein concentration using the BCA assay (Pierce) and normalized by bacterial cell numbers in CFU. DNA measurement was obtained with qPCR using *S. mutans*-specific 16S rRNA primers (Table 2).

Colocalization of glucan-eDNA in cell-free matrix formation system. *S. mutans* was grown in THB to an OD_{470} of 1.0 and centrifuged at 1,950 × *g* at 4°C for 10 min, and the cell-free culture supernatants were filtered through 0.22-μm syringe filters to remove residual cells. The cell-free supernatants were then concentrated with the addition of 100% ethanol (300 μl ml^{-1} supernatant) and frozen at −80°C for

1 h. Supernatant proteins and eDNA were harvested by centrifugation at 15,682 × *g* for 10 min at 4°C, resuspended in TYE medium supplemented with 1% sucrose and Cascade blue conjugated to dextran, added to the wells of a 96-well plate, and incubated at 37°C in 5% CO_2 for 16 h. SYTO9 was added to the wells, and the mixture was incubated for 20 min. A total of 10 μl was taken from the well and placed on a microscope slide for imaging using the 60× objective magnification on the Nikon Eclipse TE2000-E inverted fluorescence microscope.

Rat caries model. The virulence of the *S. mutans smu_833* mutant was compared with that of the WT using a rat caries model of infection (29–33, 56). Briefly, male and female rat pups (Fischer 344), 18 days of age, were weaned and divided into 2 groups of 5 animals each. Rats were challenged at 22 days of age with either wild-type *S. mutans* or the *smu_833* mutant by oral swabbing with a fresh overnight culture. Rats were provided a caries promoting diet (MIT 305) containing 5% sucrose and sterile drinking water *ad libitum*. Oral swabs were taken 5 days postinfection to confirm colonization. Rats were weighed at weaning and at the termination of the experiment. The rats were sacrificed at 60 days of age (day 38) and their mandibles excised for plaque analysis and scoring for caries by the method of Keyes (58). Bacterial CFU were enumerated from mitis salivarius (MS) agar. Statistical analysis was done using Student's *t* test. All animal studies were done according to National Institutes of Health guidelines, using protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02247-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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K.R. and H.W. designed experiments and wrote the manuscript. Z.T.W. conducted membrane vesicle quantification experiments. S.M.M. performed the rat caries study. K.R. conducted the remaining experiments. All authors reviewed the final draft of the manuscript.

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