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Inactivation of a gene for a fibronectin-binding protein of the oral bacterium *Streptococcus mutans* partially impairs its adherence to fibronectin

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ABSTRACT

A sequence of 1647 base pairs in length of *Streptococcus mutans* DNA that encodes for a 63-kDa protein with significant amino acid similarity with fibronectin-binding proteins of *Streptococcus pyogenes* and *Streptococcus gordonii* was cloned. The putative recombinant fibronectin-binding protein of *S. mutans* was purified using affinity chromatography and the cloned protein was used to prepare polyclonal antibodies against the recombinant protein. In immunoblot assays, antibodies against the *S. pyogenes* fibronectin-binding protein, FBP54, were cross-reactive with the *S. mutans* protein that was designated SmFnB. Additionally, antibodies to the *S. mutans* SmFnB protein reacted with the *S. pyogenes* FBP54 protein. The *S. mutans* SmFnB protein was found to bind to immobilized fibronectin in a concentration dependant manner. A mutant strain of *S. mutans* M51 that was constructed by allelic exchange did not express the SmFnB protein. This mutant strain, *S. mutans* ΔSmFnB, was determined in an ELISA to bind to immobilized fibronectin 30% less when compared to the parental strain *S. mutans* M51. The results are consistent with the conclusion that the 63-kDa SmFnB protein of *S. mutans* is a fibronectin-binding protein that may contribute to the interaction of *S. mutans* with damaged heart tissue during pathogenesis of infective endocarditis. Also, the study suggests that multiple molecules may mediate the interaction of *S. mutans* with fibronectin.

1. Introduction

Infective endocarditis is an infection of the cardiac tissue that occurs when various species of microorganisms attach in the form of a vegetation to cardiac tissue. Viridans streptococci account for over 50% of all infective endocarditis cases. *Streptococcus mutans*, a member of the mutans group of streptococci and historically a member of the viridans streptococci, is the causative agent of dental caries and is responsible for about 20% of the cases of oral streptococci-associated endocarditis [1]. Oral streptococci can enter the bloodstream after trauma to the oral cavity, such as a dental procedure, and attach to platelet-fibrin matrices formed on injured endothelial tissue. Certain oral streptococci have been shown to induce platelet aggregation, a trait that is believed to be crucial in the pathogenesis of streptococcal-mediated infective endocarditis [2].

The adherence of bacteria to damaged heart tissue is a significant event in the pathogenesis of subacute (chronic) infective endocarditis caused generally by oral streptococci. Adherence is mediated by structures on the bacterial surface and specific structures associated with host cells. Since the oral bacteria that cause chronic infective endocarditis primarily adhere to damaged heart tissue, it is believed that extracellular matrix molecules act as receptors for bacterial attachment. Previous studies have shown that a number of species of bacteria, including *S. mutans*, adhere to certain extracellular matrix proteins such as, fibronectin, laminin, collagen, and the blood clotting component, fibrinogen [3–9]. Fibronectin is a 440-kDa dimeric glycoprotein that is a common substrate for the attachment of bacteria to the host cell surface. There are two main forms of fibronectin, the secreted insoluble matrix protein and the soluble form present in plasma and extracellular fluids. Streptococci and staphylococci express various fibronectin-binding adhesins [7,10–14]. The binding of pathogenic *Streptococcus pyogenes* and *Staphylococcus aureus* to epithelial cells via fibronectin facilitates their entry into cells [15–17]. The structural organization of the best characterized fibronectin-binding proteins of streptococci and staphylococci is similar [18]. Proteins from both bacteria are surface proteins that have a signal peptide sequence for secretion, a LPXTG motif for cell wall anchoring, and a fibronectin-binding domain composed of several amino acid repeat sequences. Also, several species of streptococci express

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atypical fibronectin-binding proteins that do not contain a secretion signal, anchoring motif, or repeat sequences for binding to fibronectin. These atypical fibronectin-binding proteins include FBP54 of *S. pyogenes*, PavA of *Streptococcus pneumoniae*, and FbpA of *Streptococcus gordonii* [7,10,19]. Chia et al. observed that *S. mutans* cells could adsorb on their surface soluble fibronectin present in plasma [14]. Furthermore, these investigators demonstrated that *S. mutans* can bind soluble and immobilized fibronectin and identified a cell wall-associated protein, FBP-130, as a receptor that bound fibronectin. However, the gene that encodes FBP-130 is not known. In this study, we have identified a 63-kDa protein of *S. mutans* that binds to fibronectin that appears to be different from the fibronectin protein identified by Chia et al. These studies report the first cloning and characterization of a fibronectin-binding protein of *S. mutans* and support the rationale of exploring the possibility that multiple fibronectin-binding proteins expressed by *S. mutans* may facilitate adherence of *S. mutans* cells to cardiac tissue.

2. Results

2.1. Identification of a gene for a putative fibronectin-binding protein of S. mutans

The genome sequence of *S. mutans* UA159 was searched for a gene that encoded for a fibronectin-binding protein. This search yielded a sequence of 1647 base pairs that when translated generated a protein of 549 amino acids with a molecular weight of approximately 63-kDa. Sequence alignment showed that the 63-kDa protein of *S. mutans* had strong amino acid sequence homology with several known fibronectin-binding proteins. Comparison of the *S. mutans* amino acid sequence to the sequence of the fibronectin/fibrinogen binding protein of *S. pyogenes* FBP54 showed that 333 of 473 (70%) amino acids are identical and 61 are conserved substitutions indicating an overall similarity of 83% [7]. When the translated sequence of the *S. mutans* fibronectin-binding protein was compared to the fibronectin-binding protein of *S. gordonii* (FbpA), the alignment showed that 409 of 549 (74%) amino acids were identical and 70 were conserved substitutions indicating an overall similarity of 87% [10]. Similar levels of identity were found for the fibronectin-binding PavA protein of *S. pneumoniae* and the putative fibronectin-binding protein of the oral streptococcus, *Streptococcus sanguinis* [19,20]. Fig. 1 shows sequence comparisons using the Clustal W multiple sequence alignment tool of *S. mutans* (SmFnB), *S. gordonii* (FbpA), and *S. pyogenes* (FBP54) fibronectin-binding proteins.

The identified sequence that encoded for the putative fibronectin-binding protein of *S. mutans* was PCR amplified and cloned into an expression vector for purification of the protein product. Initially, the expressed protein was purified under denaturing conditions using nickel affinity chromatography. The purified rSmFnB protein was used to produce antibodies against the putative *S. mutans* fibronectin-binding protein. Western blot analysis demonstrated that the anti-SmFnB sera were able to react with purified rSmFnB protein and also with the FBP54 fibronectin-binding protein of *S. pyogenes* (Fig. 2B). Antibody against the FBP54 protein detected both the purified FBP54 protein and the purified rSmFnB protein (Fig. 2C). These results provide evidence that the SmFnB protein has antigenic determinants in common with a known fibronectin-binding protein.

2.2. Binding of the SmFnB protein to immobilized fibronectin

We wanted to demonstrate that SmFnB protein had the ability to bind to fibronectin. For these studies, the sequence of *smFnB* gene was cloned into an expression vector that allowed for the expression of the SmFnB protein fused to maltose-binding protein (MBP) and purification of the protein in a native conformation using amylose affinity chromatography. The purified MBP–SmFnB fusion protein was used in an ELISA binding assay to determine if the protein interacts with immobilized fibronectin. The assay demonstrated that the binding of the SmFnB fusion protein to immobilized fibronectin occurs in a dose-dependant manner (Fig. 3). In contrast to the SmFnB fusion protein, the interaction of maltose-binding protein with immobilized fibronectin was significantly less. This result reinforces the conclusion that the *smFnB* gene of *S. mutans* encodes for a fibronectin-binding protein.

2.3. Binding of SmFnB mutant strain to immobilized fibronectin

To determine if expression of the SmFnB protein contributes to the binding of *S. mutans* cells to fibronectin, an isogenic mutant of *S. mutans* M51 that does not express the fibronectin-binding protein was constructed by allelic exchange. The mutated strain was constructed by the removal of approximately 1100 base pairs from the central region of the gene and insertion of a DNA fragment containing a kanamycin resistance determinant within the gene. Once transformed into *S. mutans* cells, the mutated gene replaced the wild-type sequence on the chromosome by a double crossover recombinational event. To verify that the recombinational event had occurred in the mutated *S. mutans* M51 strain, chromosomal DNAs from the wild-type and mutated strains were isolated and used as a template in PCR amplification and Southern blot analysis (Fig. 4A). The results demonstrated that the kanamycin resistance cassette was inserted into the fibronectin-binding gene of the mutated *S. mutans* strain. SDS-PAGE and Western blot analysis with the total protein lysates of the wild-type M51 and ΔSmFnB strains were used to determine if the modification in the genome interrupted the expression of the 63-kDa SmFnB protein. The results showed that in the total protein lysate of the mutated strain that there was not a protein that reacted with anti-SmFnB antibodies (Fig. 4B). The ELISA technique was used to determine if the abrogation of protein expression in the mutant strain results in a decrease in binding to fibronectin. The results from the binding assay showed that binding to immobilized fibronectin by the mutant strain was reduced by about 30% when compared to the wild-type strain (Fig. 4C).

3. Discussion

The overall goal of this work was to use a genetic approach to identify and study genes of the oral streptococcus, *S. mutans*, that could play a role in the pathogenesis of infective endocarditis caused by this microorganism. Previous studies have shown that various Gram-positive bacteria express multiple receptors that bind to fibronectin and have a critical role in the infectious process [21]. However, information highlighting what role fibronectin-binding by *S. mutans* plays in the pathogenesis of infective endocarditis is sparse. Therefore, identification of genes that encode for proteins of *S. mutans* that bind extracellular matrix molecule and for proteins that induce platelet aggregation and binding to platelets will assist in understanding the mechanism by which this traditionally non-life threatening microorganism colonizes damaged endocardial surfaces.

Amino acid alignment comparisons demonstrated that the amino acid sequence of the putative fibronectin-binding protein of *S. mutans* shares significant sequence homology with the fibronectin-binding proteins of several streptococcal species. Analysis of the amino acid sequence of the *S. mutans* fibronectin-binding protein described in this study indicates that the *S. mutans* protein is a member of the atypical fibronectin-binding proteins of bacteria.

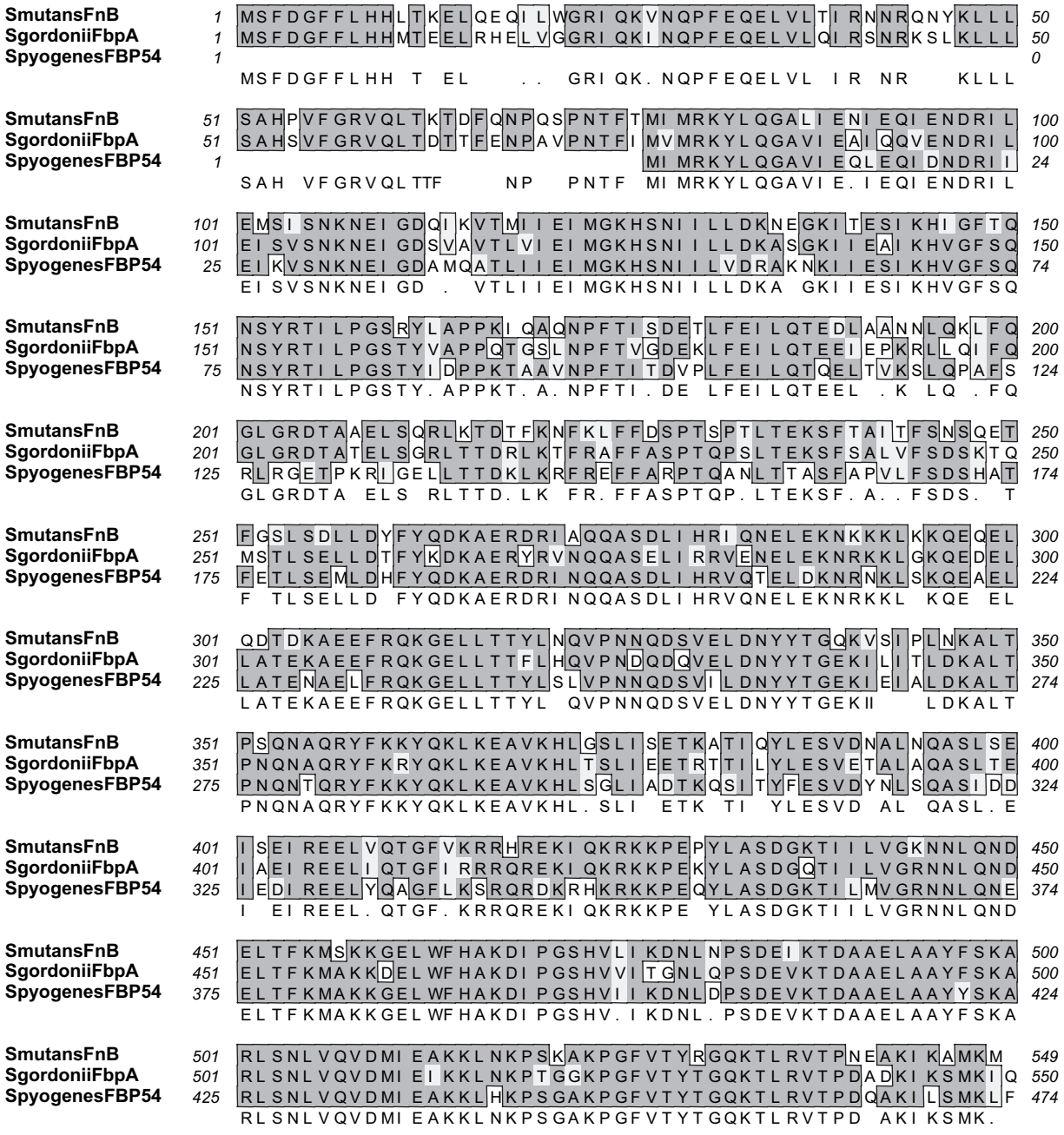

Fig. 1. Clustal W sequence alignment of the translated protein sequences of *S. mutans* (SmFnB), *S. gordonii* (FbpA), and *S. pyogenes* (FBP54) fibronectin-binding proteins.

Antibodies against the FBP54 protein of *S. pyogenes* were able to detect the rSmFnB protein of *S. mutans* and SmFnB antibodies were cross-reactive with the FBP54 protein. This finding suggests that the two proteins are immunologically similar. Previous studies have shown that the FBP54 protein protects rats from developing infection in an experimental model of infective endocarditis [22]. Future studies could be directed toward identifying common epitopes of the proteins that could be used in the development of a vaccine protective against multiple infectious organisms.

The results of this study demonstrate that the 63-kDa SmFnB protein expressed by *S. mutans* cells binds to fibronectin and this interaction could facilitate bacterial adherence to host tissue. Comparative analysis between the wild-type and the mutated SmFnB strain showed that binding of the mutant strain to fibronectin was not totally ablated. This finding is similar to that observed for a *S. gordonii* mutant strain that had a mutation in the fibronectin-binding protein, FbpA [10]. The most obvious explanation for this result is that *S. mutans* cells express multiple fibronectin-binding proteins. In support of this, Chia et al. showed that fibronectin bound to 130- and 55-kDa proteins expressed by *S. mutans*. The investigators observed that antibodies for the FBP-130 protein were not reactive against the 55-kDa protein demonstrating that another protein also binds to fibronectin. Our search did not identify a protein of 130-kDa with characteristics of a known fibronectin-binding protein. The 63-kDa protein fibronectin-binding protein identified in this study may be the same protein identified earlier by Chia et al. Therefore, further studies are required to verify that the fibronectin-binding protein identified in this study is or is not the 55-kDa protein previously described. A study by Sciotti et al. reported that the N-terminal region of antigen I/II bound to various extracellular matrix proteins such as, fibronectin, laminin and collagen [23]. Our laboratory has compared the

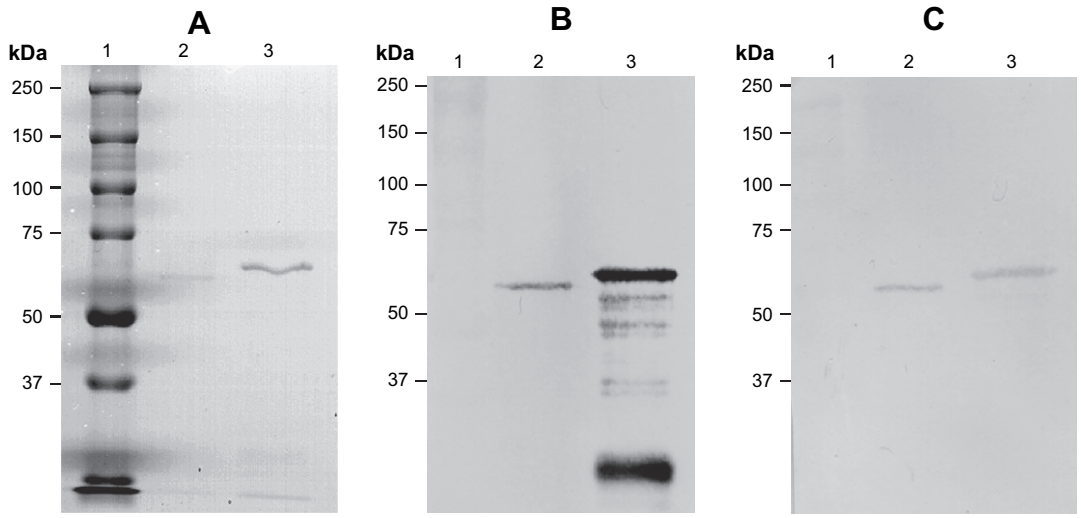

Fig. 2. Reactivity of antibodies against the *S. mutans* and *S. pyogenes* fibronectin-binding proteins with purified SmFnB protein of *S. mutans* and FBP54 protein of *S. pyogenes*. (A) SDS-PAGE of purified SmFnB and FBP54 proteins. (B) Immunoblot probed with anti-SmFnB sera. The reactive band of approximately 25 kDa in lane 3 is probably a truncated form of the recombinant SmFnB protein generated during expression of the protein in *E. coli*. (C) Immunoblot probed with anti-FBP54 sera. Lanes: (1) molecular weight markers; (2) FBP54; (3) SmFnB.

ability of parental strain, *S. mutans* NG8, and its isogenic antigen I/II-deficient mutant strain, 834, to bind fibronectin [9]. We find that the mutant NG8 strain demonstrated a decreased adherence to immobilized fibronectin. Collectively, these studies suggest that *S. mutans* has multiple fibronectin-binding proteins.

In summary, *S. mutans* cells express a 63-kDa protein that shares amino acid sequence homology with atypical fibronectin-binding proteins of several streptococcal species. A mutant strain of *S. mutans* that possessed a mutation in the gene for a fibronectin-binding possessed a reduced ability to bind fibronectin that suggests that the 63-kDa fibronectin-binding protein contributes to the ability of *S. mutans* cells to bind fibronectin and possibly adherence to host cells. It remains to be determined whether the SmFnB protein is involved in the pathogenesis of infective endocarditis.

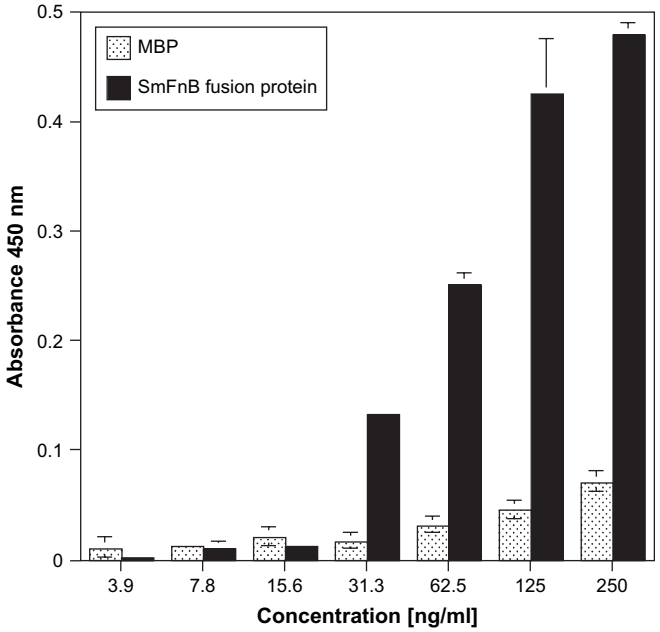

Fig. 3. Binding of recombinant SmFnB fusion protein to immobilized fibronectin. The wells of microtiter plates were coated with fibronectin and reacted with various concentrations of SmFnB fusion protein and maltose-binding protein (control). Absorbance values represent means of at least triplicate samples and are representative of three experiments.

4. Materials and methods

4.1. Bacterial strains and media

S. mutans M51 has a spontaneously generated streptomycin-resistance phenotype and was obtained from the Centers for Disease Control and Prevention and was isolated from the peripheral blood of a patient clinically diagnosed with infective endocarditis [9]. The isogenic mutant of *S. mutans* M51, ΔSmFnB, was constructed by allelic exchange and has a deficiency in the expression of the SmFnB protein. *S. mutans* M51 and the isogenic ΔSmFnB strains were anaerobically grown at 37 °C in Todd-Hewitt broth in the presence of 500-μg/ml of streptomycin or 500-μg/ml each of streptomycin and kanamycin, respectively. *Escherichia coli* TG1 cells that were used to maintain the recombinant pQE31 constructs were grown in LB media containing kanamycin and ampicillin at a concentration of 25-μg/ml and 100-μg/ml, respectively. *E. coli* M15 (pREP4) cells that were used to express the SmFnB protein cloned into the his-tagged expression vector pQE31 and the *E. coli* BL21Star (DE3) strain that contains the recombinant pMAL-c2x vector with the inserted *smFnB* gene were grown in LB media containing 100-μg/ml ampicillin.

4.2. Isolation of plasmid DNA and S. mutans chromosomal DNA

Chromosomal DNA of *S. mutans* strains was isolated using the method previously described [24]. Plasmid DNA was isolated according to the Qiagen plasmid DNA protocol. The PCR products were isolated and purified according to the protocol from the Wizard PCR preps purification kit (Promega).

4.3. Cloning of the gene for a fibronectin-binding protein of S. mutans

The DNA sequence for a putative fibronectin-binding protein of *S. mutans* was obtained from a BLAST search of the *S. mutans* genome sequence ([25]; GenBank accession no. AE014133). To clone the DNA sequence that encoded the putative fibronectin-binding protein, the sequence was amplified with Taq DNA polymerase by PCR using the primer SM 10 [5′-ATGTCTTTTGATGGCTTTTTTTTA-CAT-3′ (forward primer; bases 1–27 of the sequence)] and SM 11 [5′-TTACATCTTCATCGCTTTTATCTTAGC-3′ (backward primer, bases

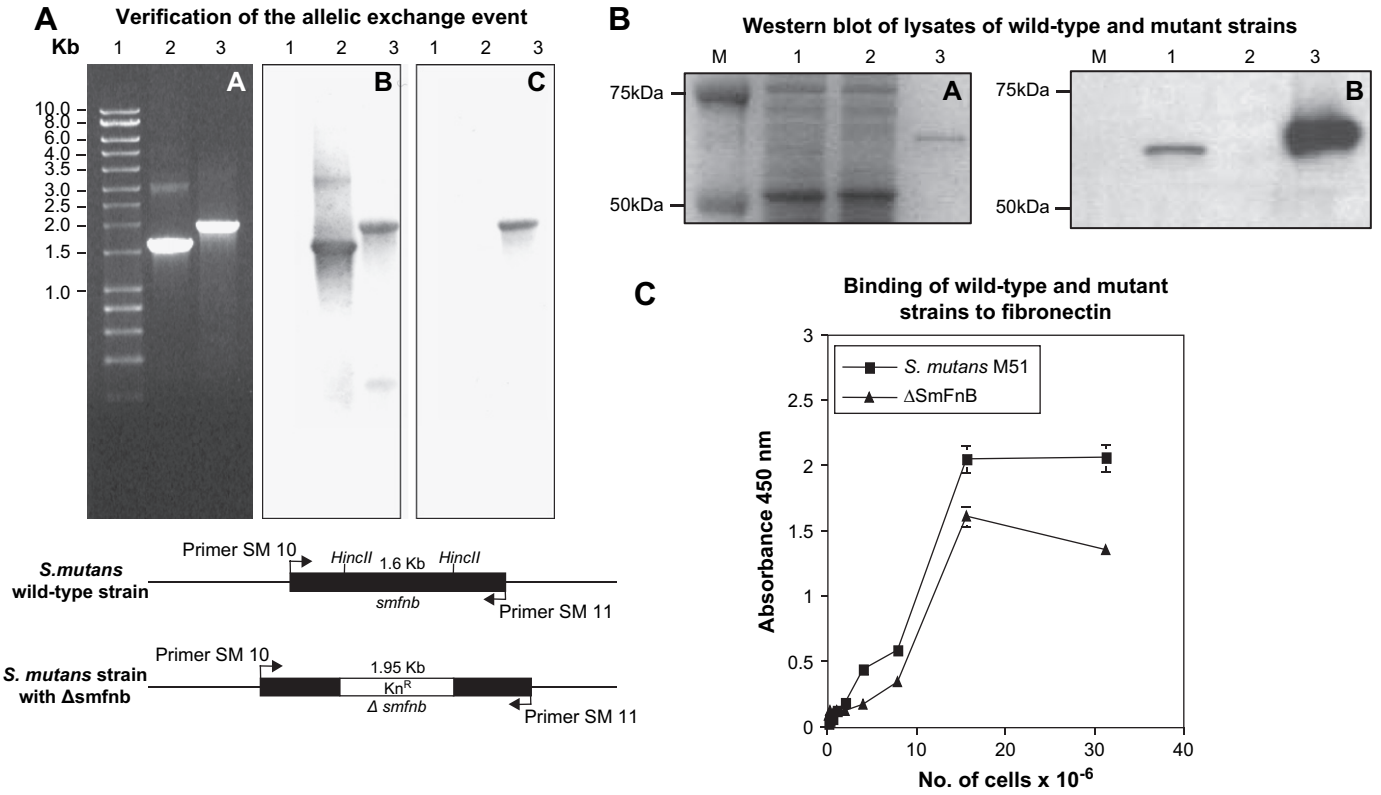

Fig. 4. Properties of strain with the mutated *smFnB* gene. Verification of the allelic exchange event by PCR amplification and Southern blot analysis (Panel A). Procedures were performed as described in Section 4. Lane 1, 10-kb DNA ladder; Lane 2, wild-type *S. mutans* chromosomal DNA used as template in PCR reaction; and Lane 3, mutated *S. mutans* chromosomal DNA used as template in PCR reaction. (A) Southern blot of PCR reactions probed with digoxigenin-labeled 1.65-kb fibronectin-binding gene of *S. mutans*. (B) Southern blot of PCR reactions probed with digoxigenin-labeled 1.4-kb fragment containing kanamycin resistance gene. SDS-polyacrylamide gel electrophoresis and Western blot analysis of *S. mutans* wild-type and ΔSmFnB total protein lysates (Panel B). (A) Total protein lysates separated electrophorectically on an 8% SDS-polyacrylamide gel. (B) Immunoblot of gel probed with affinity purified anti-SmFnB sera. Lanes: (M) molecular weight marker; (1) lysate from wild-type cells; (2) lysate from ΔSmFnB cells; (3) purified his-tagged SmFnB protein. Binding of *S. mutans* M51 wild-type and the isogenic mutant ΔSmFnB to immobilized fibronectin (Panel C). Values represent means of at least triplicate samples and are representative of three experiments.

1624–1650 of the sequence and includes termination codon)] and *S. mutans* M51 chromosomal DNA as template.

The 1650 base pair product was then cloned into the T/A cloning vector pCR T7/NT TOPO TA and transformed into *E. coli* One Shot TOP10 cells and transformants were selected on LB agar plates containing 50-μg/ml ampicillin. The DNA of transformants was isolated and digested with BamHI and HindIII restriction enzymes to release inserted DNA. The insert DNA was gel purified and then cloned into BamHI and HindIII digested expression vector pQE31. For stable expression of the histidine tagged SmFnB protein, plasmid pQE31(SmFnB) was transformed into *E. coli* M15 (pREP4) cells. The *S. mutans* fibronectin-binding protein that we have designated SmFnB is assigned the name SMU.1449 in the published *S. mutans* genomic sequence [25].

Cloning of the *smFnB* gene into the maltose-binding protein fusion vector, pMAL-c2x, was accomplished by amplifying the gene with Pfx DNA polymerase using the primer pair SM 10 and SM 11 and *S. mutans* genomic DNA as template. The amplified PCR product was purified and then ligated into XmnI linearized pMAL-c2x vector (New England Biolabs). Plasmid DNA was isolated from transformants and the presence of insert DNA was verified by restriction enzyme digestion and DNA sequence analysis.

4.4. Expression and purification of S. mutans fibronectin-binding protein

Recombinant *S. mutans* fibronectin-binding protein (rSmFnB) was purified from 1-l cultures induced and processed using denaturing conditions as described previously using the Qiagen protocol [26]. To determine the purity of eluted protein, samples collected from the metal affinity column were separated on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane and probed with mouse anti-His antibodies. Detection of reactive protein bands was achieved by incubation of membranes with alkaline phosphatase conjugated goat anti-mouse IgG followed by a substrate combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide.

For purification of the MBP–SmFnB fusion protein, 1-l cultures were induced with IPTG and the cell pellet was suspended in column buffer (20-mM Tris–HCl, pH 8.0, 200-mM NaCl and 1-mM EDTA) and lysed by sonication. The cell extract containing the fusion protein was loaded onto an amylose resin column, washed with column buffer, and the fusion protein was eluted with buffer containing 10-mM maltose. The fractions containing purified fusion protein were detected by SDS-PAGE and Western blot analysis using anti-MBP serum (New England Biolabs).

Total cell lysates of *S. mutans* M51 wild-type and the ΔSmFnB strains were prepared from 20-ml cultures. Cells in suspension were lysed using a Mini-Beadbeater 3110BX and 0.4-mg of 0.1-mm zirconia/silica beads per ml of cell suspension. After cell lysis, the beads were allowed to settle and the supernatant fluid (total cell extract) was removed.

4.5. Preparation of antiserum to the purified SmFnB protein

Antibodies against the purified recombinant SmFnB protein were raised in New Zealand white rabbits by subcutaneous

injections of 80-μg of protein emulsified in Freund's incomplete adjuvant containing muramyl dipeptide. Rabbits were boosted 3 weeks after the initial injection with 40-μg/ml of the rSmFnB protein in Freund's incomplete adjuvant. After an additional 2 weeks, the animals were bled and the immunoglobulin fraction of the serum was isolated. Antibodies used to obtain the results in Fig. 4B were further purified by affinity chromatography on a column containing purified rSmFnB coupled to Sepharose 4B beads.

4.6. Detection of rSmFnB protein with antibodies for the S. pyogenes fibronectin-binding protein, FBP54

Purified *S. pyogenes* FBP54 protein and anti-sera against this protein were obtained from Harry Courtney [7]. Purified rSmFnB and *S. pyogenes* FBP54 proteins were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Detection of the *S. mutans* and *S. pyogenes* fibronectin-binding proteins fixed on the nitrocellulose membrane was performed by separately incubating each blot for 1-h with rabbit anti-FBP54 and anti-SmFnB sera and processing the blots as described above using alkaline phosphatase conjugated goat anti-rabbit IgG.

4.7. Construction by allelic exchange of S. mutans ΔSmFnB strain

To construct a fibronectin-binding deficient strain of *S. mutans* M51, 1054 base pairs of the *smFnB* gene were removed by restriction enzyme digestion with HincII. A 1394 base pair kanamycin resistance gene residing on the plasmid pVA2592 was amplified by PCR using the primer pair 5′-GATAAACCCAGCGAACCATTTG-3′ (forward primer) and 5′-GGATCCCGAGCTTTTTAGAC-3′ (backward primer) [27]. The amplified kanamycin resistance gene was inserted into the deleted sequence of the *smFnB* gene in the recombinant pQE31 construct. The modified sequence was then PCR amplified with the primer pair SM 10 and SM 11 and *S. mutans* M51 cells were transformed with the PCR product as described by Perry and Kuramitsu and kanamycin resistant transformants were selected [28]. The presence of the kanamycin resistance determinant in the fibronectin-binding gene of *S. mutans* M51 was verified by PCR amplification and Southern blot analysis.

4.8. PCR and Southern blot analysis verifying the insertion of the kanamycin resistance cassette into the mutated smFnB gene of S. mutans that encodes for a fibronectin-binding protein

The insertion of the kanamycin resistant cassette into the deleted sequence of *S. mutans* that encodes for the fibronectin-binding protein was verified by PCR using primers SM 10 and SM 11. The PCR amplified products were ran on a 0.8% agarose gel and transferred to a nylon membrane by capillary transfer. The blots were probed with a digoxigenin-labeled 1.65-kb DNA fragment containing the *smFnB* gene of *S. mutans* M51 and a 1.4-kb fragment containing the kanamycin resistance cassette according to the manufacturers protocol (Roche Molecular Biochemicals).

4.9. Fibronectin-binding assays

To evaluate the ability of the recombinant SmFnB fusion protein to bind fibronectin, an ELISA was utilized. Human plasma fibronectin was immobilized onto microtiter plate wells by adding 100 μl of the protein solution (10-μg/ml in 0.05-M carbonate buffer, pH 9.6) to each well and incubating for 16-h at room temperature. After blocking for 1-h at 25 °C with 10-mM PBS containing 0.05% BSA and 0.025% Tween 20, various concentrations of the SmFnB–MBP fusion protein were added and the plates were incubated for 1-h at room temperature. Purified maltose-binding protein was used as a control and was added to the plate in a similar manner as the SmFnB fusion protein. After washing, the plates were incubated for 1-h at room temperature with antibodies against maltose-binding protein. The plates were washed and 100-μl of goat anti-rabbit IgG conjugated with horseradish peroxidase was added to each well. The plates were developed with the chromogenic substrate tetramethylbenzidine and the absorbance read at 450-nm. The ELISA technique previously described was used to measure the comparative ability of wild-type *S. mutans* M51 and the isogenic strain with the mutated gene for the fibronectin-binding protein to bind to fibronectin [9].

Acknowledgments

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