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Identification and characterization of collagen-binding activity in *Streptococcus mutans* wall-associated protein: A possible implication in dental root caries and endocarditis

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Abstract

Streptococcus mutans is implicated in coronal and dental root decay, and in endocarditis. Comparative study of the amino acid sequence of *S. mutans* 47 kDa wall-associated protein A (WapA) revealed a collagen-binding domain (CBD) at the N-terminal region. Recombinant AgA (WapA truncated at the carboxyterminal end) was isolated, biotin-labeled, and analyzed by Solid Phase Binding Assay. The results showed that biotin-labeled AgA bound significantly and in a dose-dependent manner to immobilized collagen type I, and to a lesser extent to fibronectin, but not to collagen type IV or laminin. Binding of biotin-labeled *S. mutans* cells to collagen-coated surfaces was significantly inhibited by antibody to WapA or AgA ($P < 0.001$). The results obtained confirmed the collagen-binding activity of CBD in AgA and WapA, and suggested that WapA may be used, not only as a vaccine against coronal and dental root caries, but also against *S. mutans*-mediated endocarditis.

Keywords: Collagen-binding activity; Dental root decay; Endocarditis; *Streptococcus mutans*; Wall-associated protein A

The role of *Streptococcus mutans* in coronal caries involves tooth colonization and buildup of dental plaque, which are mediated by surface antigens binding to salivary glycoproteins [1], glucan-binding proteins [2], and bacterial coaggregations [3]. *S. mutans* has also been found in dental root caries in ultrastructural studies [4]. The role of *S. mutans* in dental root decay was further supported by their ability to bind [5] and degrade [6] collagen type I, a major component of dentin. In addition to the well-documented binding activity to salivary pellicle via the surface antigen I/II, *S. mutans* was able to bind a wide range of extracellular matrix (ECM) molecules such as type I collagen, laminin, keratin, and fibronectin [7,8]. *S. mutans* has also been reported to colonize heart valves and endocardium causing subacute bacterial endocarditis when these organisms gain entry into the bloodstream. Crystallographic analysis of bacterial collagen-binding domains (CBDs) revealed two evolutionarily unrelated families, which were referred to as clostridial and staphylococcal CBDs [9,10]. Clostridial CBDs are conserved components of bacterial collagenases, whereas staphylococcal CBD is found in *Staphylococcus aureus* surface adhesins with binding affinity to eukaryotic collagen [9,10]. The present report is on the identification and analysis of *S. mutans* WapA CBD and collagen-binding activity.

Materials and methods

Bacterial strains and growth conditions. Streptococcus mutans strain GS-5 (serotype c) was initially obtained from J.J. Ferretti (University of Oklahoma), propagated and maintained in our laboratory stock strains. *S. mutans* was cultured in brain heart infusion (BHI) (Fisher Chemical Co., Cincinnati, OH) broth at 37 °C without agitation or on BHI agar containing 1.5% agar at 37 °C in the presence of 10% CO_2.

Specificity and localization of collagen-binding domain. The nucleotide sequence of *wap*A and its deduced amino acid sequence with NCBI's protein–protein BLAST (blastp) analysis indicated that it

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contained a sequence specific CBD. Further analysis on the specificity and localization of the collagen-binding domain of the WapA was performed with different functional domain algorithms including Position-Specific Iterated and Pattern-Hit initiated BLAST (PSI- and PHI-BLAST) and pfscan which is a method of profile analysis against PROSITE or Protein Families (Pfam) database. Confidence levels of hits against all databases used were determined based on Expectation values (*E* values) and the *E* value less than 0.001 was considered to be significant.

Structure prediction of collagen-binding domain in WapA. In order to investigate the biochemical function of the putative collagen-binding domain, secondary structure analysis was carried out using the 3D-PSSM program [11] for the amino acid sequence of the putative CBD in the WapA. The predicted secondary structure was matched against the 3D-PSSM fold library (http://www.sbg.bio.ic.ac.uk/~3dpssm/), which contains solved crystal structures. The protein sequence was also scanned against the SCOP (Structural Classification of Proteins) database, where protein domains are classified into four levels: family, superfamily, fold, and class [12].

Cloning, expression, and purification of recombinant AgA. To obtain recombinant AgA, an 885 bp DNA fragment of *wap*A containing putative collagen-binding domain but lacking the promoter, signal sequence, and carboxyterminal region was cloned into the pGEX-6P-1 glutathione *S*-transferase (GST) fusion vector (Invitrogen, Carlsbad, CA) and hosted in *Escherichia coli* BL21 (DE3). Recombinant AgA was overexpressed by inoculating 5 ml of overnight culture into 500 ml of 2× YT medium containing 16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter, pH 7.0, supplemented with 100 μg/ml ampicillin. The culture was incubated at 37 °C with shaking until one OD_{600} was reached, at which time, IPTG was added at a final concentration of 1 mM in order to induce cloned gene expression. At 6 h post-incubation, the cells were harvested by centrifugation at 7700*g* for 10 min at 4 °C, and washed with 250 ml cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor, followed by 1 min sonication on ice and centrifugation at 12,000*g* for 30 min. Solubilization of the fusion protein was performed by mixing with 20% Triton X-100 for 30 min. After centrifugation at 12,000*g* for 10 min at 4 °C, the supernatant was incubated on a rocker for 30 min at room temperature with 2 ml of the 50% slurry of glutathione–Sepharose 4B (Pharmacia). The mixture was transferred onto the Sepharose column, and the column was washed with 15 ml PBS. After the column was washed with 2.5 ml of PreScission (Amersham, Piscataway, NJ) cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.0), the PreScission Protease mix containing 20 μl of PreScission Protease with 230 μl of PreScission cleavage buffer was loaded onto the column and incubated on a rocker at 5 °C for 4 h to cleave off the GST tag. The unbound AgA was eluted with 800 μl of PreScission cleavage buffer and analyzed by SDS–PAGE.

Biotin labeling. Biotin labeling of the purified AgA or *S. mutans* GS-5 cells was performed with Sulfo-NHS-LC-Biotin reagents (Pierce, Rockford, IL) as follows. The AgA or GS-5 cells in PBS (pH 8.0) were mixed with a 20-fold molar excess of 10 mM sulfo-NHS-biotin reagent solution with the protein, and the mixture was incubated at room temperature for 30 min. To remove free biotin and reaction byproducts, the reaction solution containing biotin-labeled rAgA was dialyzed against three changes (4 L each) of PBS (pH 8.0) overnight using dialysis tubing with a nominal Molecular Weight CutOff (MWCO) 12,000–14,000. Free biotin reagent and byproducts were removed by washing three times with PBS (pH 8.0).

Dot blot collagen-binding assay. To test the collagen-binding ability of AgA, a soluble native bovine type I collagen (Sigma, St. Louis, MO) was diluted in PBS at 5 μg/ml and was dotted (10 μl per dot) onto a piece of nitrocellulose membrane. Bovine serum albumin (BSA), 5 μg/ml, was dotted and tested in parallel as a negative control. The membrane was blocked by incubation with 5% skimmed milk in PBS for 1 h, then biotin-labeled AgA was added and the incubation was continued for another 1 h at room temperature. After extensive washes with PBS-T (PBS with 0.05% Tween 20), the bound protein was detected by incubation with alkaline phosphatase-conjugated streptavidin (AP-streptavidin) (Sigma) diluted 1:1000 in PBS for 1 h at room temperature. After a final wash in PBS-T, the membrane was stained with a chromogenic substrate solution consisting of *O*-dianisidine tetrazotized (0.25 mg/ml) and β-naphthyl acid phosphate (25 mg/ml) in sodium borate buffer (pH 9.7) containing $MgSO_4 \cdot 7H_2O$ added at 1.2 mg/ml [13].

Solid phase-binding assay. To further evaluate the binding abilities of AgA to an immobilized native type I collagen and other ECM proteins, an ELISA was carried out using different concentrations of AgA. ECM proteins (Sigma, St. Louis, MO), including Native type I collagen, type IV collagen, fibronectin, and laminin, which were diluted in PBS at a concentration of 5 μg/ml, and used to coat the wells (50 μl volume per well) of 96-well microtiter plates (Immobilon IV from Dynex, Chantilly, VA) overnight at 4 °C. After rinsing of the plate with PBS-T, the wells were blocked with 200 μl of 10% skimmed milk in PBS containing 0.02% sodium azide for 1 h at room temperature. 50 μl of various dilutions (1–8 μg) of biotin-labeled purified AgA was added separately to the wells and the plate was incubated at room temperature for 1 h, followed by an extensive wash in PBS-T and incubation with AP-Streptavidin (1:1000 in PBS) for 1 h at room temperature. After a final extensive wash in PBS-T, OD_{405nm} of each well was recorded using a 680 ELISA Microplate Reader (Bio-Rad, Hercules, CA) against a PBS-coated well used as a blank. Wells coated with BSA served as a control for nonspecific binding. Relative binding was measured by monitoring the absorbance at 405 nm following the alkaline phosphatase reaction and plotted as a function of AgA concentration. Data points represent the means of OD_{405nm} values + standard deviation from three independent experiments.

Preparation of rabbit polyclonal antiserum against WapA and AgA. Polyclonal antibodies against the purified GST-tagged AgA or full-length WapA were raised in rabbits for bacterial inhibition assay. Six 4-kg female albino rabbits from the New Zealand strains (Harlan, Indianapolis, IN) were immunized intramuscularly with 100 μl of 1 mg/ml of each protein antigen emulsified in 100 μl TiterMax Gold (Sigma) adjuvant at 3-week intervals. Blood was collected before immunization and one week after each booster injection. The serum was separated and extensively adsorbed with *E. coli* and GST antigens as previously described to remove non-specific antibody [14].

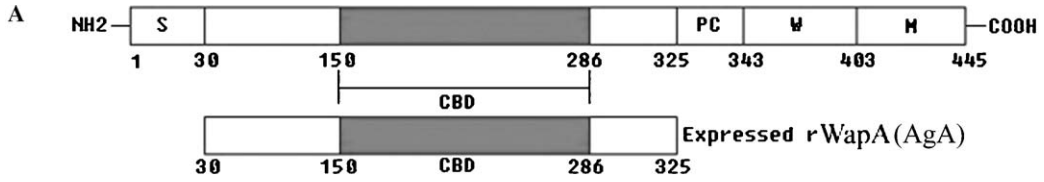
Bacterial adherence assays. To study the inhibition of collagen-binding activity by antiserum to AgA or full-length WapA, log-phase *S. mutans* cultures (OD_{600nm} of 1) were biotin-labeled and preincubated with antisera for 30 min at room temperature. The labeled cells were then transferred to microtiter plates coated with 1 μg of type I collagen per well, and the plates were incubated for 1.5 h at 37 °C, followed by washing in PBS-T. Bound cells were subsequently detected by incubation with AP-Streptavidin (1:1000 in PBS-T, containing 5% BSA), washed with PBS-T and stained for AP activity using 100 μl *p*-Nitrophenyl phosphate (pNPP) substrate at a concentration of 1 mg/ml in 1 M diethanolamine buffer, pH 9.8, per well, and the OD_{415nm} was recorded using an ELISA Reader. All samples were analyzed in triplicate.

Statistical analysis. One-way analysis of variance followed by Bonferroni test was used for multiple comparisons.

Results

Comparative sequence analysis of the putative CBD in WapA

Analysis of the deduced amino acid sequence of WapA using PSI- and PHI-BLAST revealed significant hits ($E < 0.001$) to CBD. BLASTP search against the NCBI Conserved Domain Database (CDD) showed 100% alignment at amino acid position of 150–286 of WapA to



B

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WapA:          KVGYQDKSNPGLIHWKVLINNKQGAIDNLTLTDVVGEDQEIVKDSLVAARLQYIAGDDVD 60
               : :  : ..  ::.: : :::    :.: .:::..:  : .: ::. . .:.:     :
Consensus:     KSGDVDDTDNELIRWFVNINNALQPIENAVLTDTIGLGQTLVFDSIEVYELNYKIDGTVG 60

WapA:          SLDEAASRPYAEDFSKNVTYQTNDLGLTTGFTYTIPGSSNNAIFISYTTRLTSSQSAGKD 120
               . :.. :. :    ::..   :..     ::  :. :. .:...::::: .:.. .  ..
Consensus:     QGDDVLSE-YPG--SKSIDEATDN-----GFKVTFGGTIDNSVWISYTTTITDG-GQKQS 120

WapA:          VSNTIAISGNNIN  134
               .:: ...:.::.
Consensus:     YKNTATLNGDNID  134
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C

ACCESSION #	PROTEIN PRODUCT	% Identity	*E*-value	Organism
YP_039349	Collagen adhesion protein	21.2%	2e-23	*Bacillus thuringiensis*
YP_031445	Collagen adhesion protein	21.2%	4e-23	Bacillus anthracis
AAP08047	Collagen adhesion protein	24.7%	1e-22	*Bacillus cereus*
NP_266840	Collagen adhesion	25.2%	8e-22	*Lactococcus lactis*
AAO43099	Collagen-binding protein	18.9%	3e-19	*Streptococcus equi*
AAO43108	Collagen-binding protein	12.5%	4e-19	*Arcanobacterium pyogenes*
BAD13529	Collagen-binding adhesion	14.2%	7e-19	*Streptococcus mutans Z1*
AAD43342	Collagen adhesin Ace	14.3%	4e-16	*Enterococcus faecalis*
1AMX	Collagen-Binding Domain	16.7%	1e-10	*Staphylococcus Aureus*

Fig. 1. Alignment of deduced amino acid sequences of WapA with conserved domain databases. (A) The amino acid sequence from residues 150 to 286 of WapA was aligned 100% with 134 consensus residues of collagen-binding domain in NCBI Conserved Domain Database (CDD). WapA signal peptide (S), proteolytic cleavage site (PC), cell wall domain containing LPSTG motif (W), and membrane-spanning domain (M) are indicated. The shaded region denotes the putative collagen-binding domain (150–286) that was represented by CBD. The region of the recombinant AgA, WapA truncated at the carboxyterminal end, used in this study is also shown. (B) Sequence identity ($E = 9e{-}20$) to the collagen-binding domain family was identified within the pfam database (pfam05737), where 33.8% identity in 134 amino acid overlap. (C) PSI-BLAST Multiple Sequence Alignment revealed corresponding protein products in other microorganisms.

pfam05737 consensus residues of the conserved bacterial CBD (Fig. 1).

Structure analysis of putative collagen-binding domain in WapA

Results of a secondary structure prediction algorithm using the 3D-PSSM program suggested that the submitted portion of WapA (residues 143–303) was a CBD with strong similarity with the *S. aureus* collagen adhesin Cna (PDB id: 1amx) (Fig. 2). Fold-recognition search analysis against SCOPE database defined the putative CBD of WapA to the all-β protein class and CBD of adhesin family, and recognized its structural correspondence to Cna protein of *S. aureus* with over 95% confident hit ($E = 1.68e{-}08$).

Expression of recombinant collagen-binding domain

Recombinant AgA was purified as described above and analyzed by SDS–PAGE. One band with the anticipated molecular mass of 29 kDa was obtained (Fig. 3).

Collagen-binding properties of AgA

Incubation of biotin-labeled AgA with a piece of nitrocellulose containing a dot of native type I collagen or BSA, followed by detection by incubation with AP-streptavidin and staining for AP, showed positive activity with Type I collagen dot, but not with BSA dots (Fig. 4).

Quantitative analysis of collagen-binding property of AgA

Quantitative determination of the binding activity of AgA to ECM proteins immobilized onto ELISA plates showed a dose-dependent binding to immobilized collagen type I, and to a lesser extent to fibronectin. No apparent concentration-dependent binding was observed to either laminin or BSA (Fig. 5).

Bacterial inhibition of collagen binding by antiserum

Preincubation of biotin-labeled AgA or whole cell *S. mutans* with rabbit antisera specific for AgA or WapA showed a significant blocking of collagen type I-binding

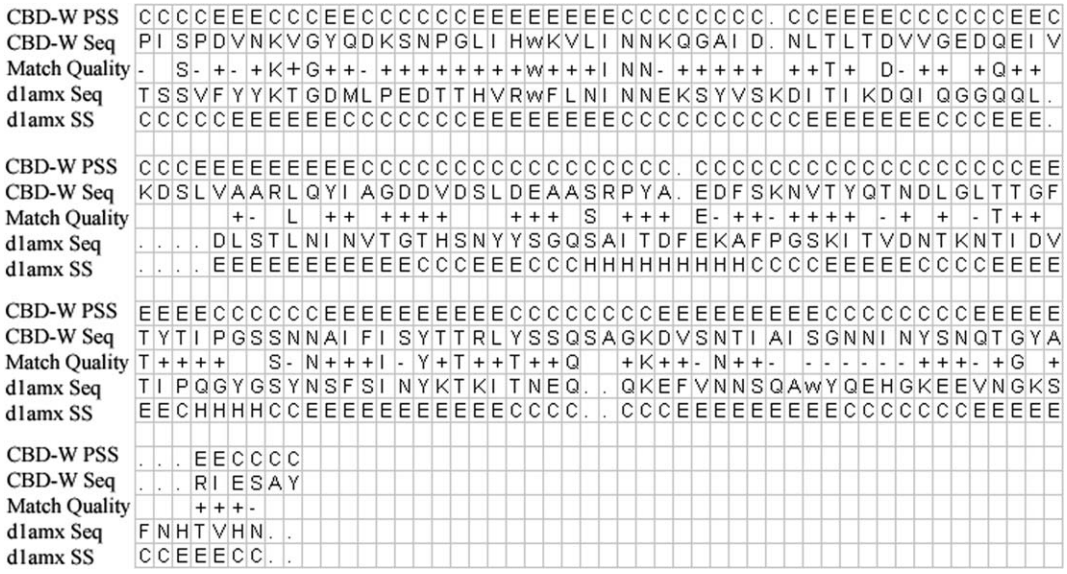

Fig. 2. Predicted secondary structure for the putative collagen-binding domain in WapA was generated using the 3D-PSSM program and was aligned to the significant structural match ($E = 1.68e{-}08$) to the polypeptide chain of the collagen-binding domain of the Cna protein from *St. aureus* from the fold library. E is extended β-strand; C is coil; and H is α-helix.

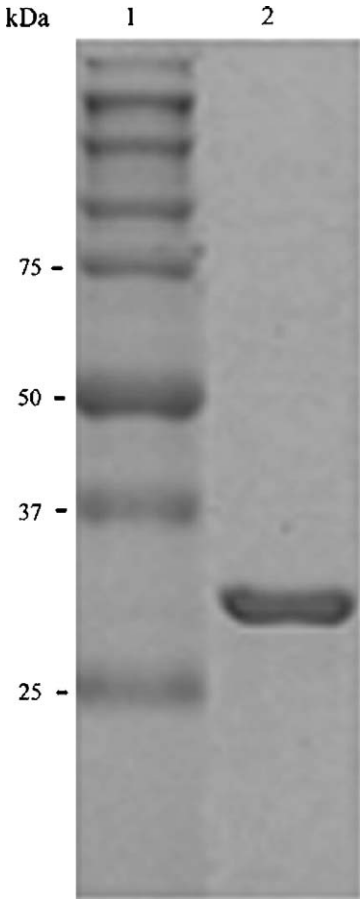

Fig. 3. SDS–PAGE analysis of purified recombinant AgA. SDS–PAGE analysis on 10% gel showed one protein band with the anticipated molecular mass of 29 kDA (lane 2). Molecular weight standards were separated in lane 1.

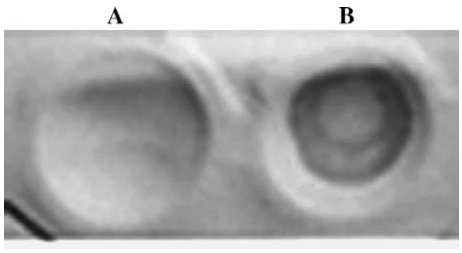

Fig. 4. Collagen-binding assay. Purified recombinant AgA only bound to the dot containing collagen type I (B) and not to dot containing BSA (A).

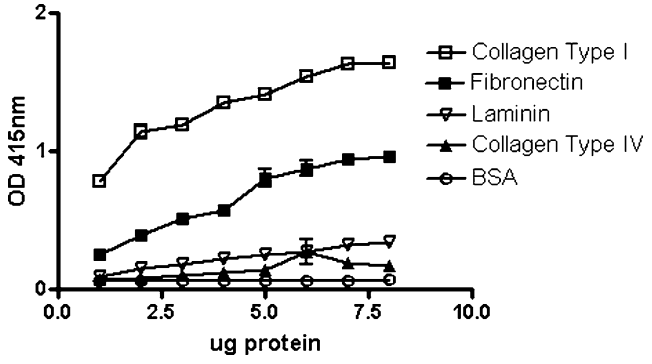

Fig. 5. Binding of recombinant AgA to immobilized ECM proteins. Immobilized ECM proteins, type I and type IV collagen, fibronectin, and laminin, were incubated with various concentrations (1–8 μg in 50 μl PBS) of biotin-labeled AgA. Wells coated with BSA were used as negative controls. Bound protein was detected by AP-streptavidin and the absorbance at 405 nm was recorded. Data are plotted as means ± standard deviation values of triplicate assay.

Table 1
Inhibition activity of antiserum on bacterial attachment to collagen type I

Pretreatment of GS-5 cells[a]	Relative adherence expressed as OD of biotin-labeled GS-5 cells ± SD[b]	Mean % inhibition[c]
None	1.633 ± 0.2838	
Pre-immune serum	1.343 ± 0.1304	17.7
Anti-AgA serum	0.5110 ± 0.08937	68.7
Anti-fWapA serum	0.2577 ± 0.01168	84.2

[a] The biotinylated *S. mutans* GS-5 was pre-incubated with antiserum before it was added to collagen type I coated microtiter wells.
[b] Each value represents the means of OD_{415nm} of biotinylated-bound cells and its standard deviation values derived from triplicate experiments.
[c] The percent inhibition was calculated by percent OD of mean difference of control and antiserum pretreated cells.

activity ($P < 0.001$). Preincubation of samples with preimmune antisera had no significant effect ($P > 0.05$) (Table 1).

Discussion

Collagen-binding and collagen-degrading activity of *S. mutans* have been reported previously and implicated in the pathogenicity of dental root surface caries and infectious endocarditis as collagen is the major constituent of dentin and heart valves [6,15]. A number of surface-associated and secreted proteins promote the attachment and colonization of host tissues by *S. mutans* and as such have been considered as major virulence factors and vaccine candidates to prevent this infection. WapA, a precursor to the extracellular AgA, has been shown to contribute to the tight glucan-mediated attachment of these bacteria to tooth surface and buildup of dental plaque. Both AgA and WapA are expressed in cariogenic *S. mutans* infecting humans (serotypes c, e, and f) and have been considered as promising candidate antigens in dental caries vaccine [16,17]. However, a collagen-binding protein has not yet been isolated from *S. mutans*.

WapA comprises a 29 amino acid hydrophobic signal peptide at the N-terminus, a protease cleavage site in the region of residues 325–343 where there are four closely spaced arginine and one lysine residues, and a hydrophobic wall-spanning region followed by a membrane anchor regions with an LPSTG motif located in the C-terminal region [18]. In the present study, comparative sequence analysis identified 134-amino-acid collagen-binding domain from residues 152 to 286 in *S. mutans* WapA. Further structural analysis using 3-PSSM demonstrated that the putative N-terminal collagen-binding protein of WapA shared comparable secondary structure topology with the collagen-binding domain of the Cna protein from *S. aureus*, suggesting a common function and implying a probable common evolutionary origin. Analysis of the deduced amino acid of other known surface proteins of *S. mutans*, including Pac, FruA, DexA, and GbpC, in search of functional domains did not show any CBD in these adhesins. Thus, the putative CBD in WapA may very well be a primary, structurally conserved adhesin responsible for the collagen-binding activity of *S. mutans*.

Considering the solubility of AgA versus WapA and the presence of a CBD in the N-terminal region, AgA was used in binding assays to collagen and other ECM proteins. Significant binding to collagen type I, and binding to fibronectin to a lesser extent, was observed, suggesting that WapA may be a surface adhesin with dual-binding affinity to glucan and ECM proteins, especially collagen type I. Thus, in addition to its role in coronal caries, WapA may play a role in *S. mutans* colonization of dentin in dental root that is exposed due to gum recession or injury. In support of WapA contributing to dental root decay is our detection of collagen fibril degradation by *S. mutans*. Two putative collagenases of the peptidase/collagenase U32 family have been cloned and currently analyzed in our laboratory. Interestingly, no CBD was identified in the deduced amino acid sequence of these proteins (manuscript in preparation). Thus, the presence of CBD in WapA indicated dual adesin functions for this protein, which include dextran- and collagen-binding activities.

Streptococcus mutans has been commonly found in endocarditis, a condition that may be contributed by the ability of these bacteria to bind fibronectin and collagen in host tissues. The mechanisms, by which *S. mutans* causes the disease, have not yet been established as no factor with these properties has been characterized. Data obtained in the present study support further analysis of WapA as a putative pathogenic factor in infective endocarditis. In vitro inhibition of *S. mutans* binding properties to collagen type I by anti-AgA and anti-WapA suggested that the use of WapA in a dental caries vaccine may also confer protection against dental root decay and bacterial endocarditis by this organism, especially that WapA is widely conserved in human pathogenic strains of *S. mutans*.

Acknowledgments

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