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## ABSTRACT

A previously unidentified 120-kDa protein was detected in *Streptococcus mutans* strain Z1 and was involved in the cold-agglutination of the strain. We have identified the gene, designated *cnm*, as being involved in the agglutination of strain Z1 following random mutagenesis. The amino acid sequence of the deduced Cnm protein exhibited high similarity to those of collagen-binding adhesins from staphylococci and other organisms. To confirm whether the protein is involved in collagen-binding, we cloned a *cnm* gene fragment, overexpressed it in *E.coli*, and prepared crude extracts. The extracts containing recombinant protein exhibited binding to immobilized collagen and laminin but not to fibronectin. Compared with the parental strain Z1, the cold-agglutination-negative mutant 05A02 exhibited reduced binding to collagen and laminin but retained that to fibronectin. This gene was detected in some strains of *S. mutans*. Therefore, the *cnm* gene encoded a new strain-specific member of the collagen-binding adhesin family.

**KEY WORDS:** *Streptococcus mutans*, collagen-binding adhesin, streptococcal endocarditis, strain-specific gene.

# *Streptococcus mutans* Strains Harboring Collagen-binding Adhesin

## INTRODUCTION

*Streptococcus mutans* is regarded as the primary etiologic agent of human dental caries and resides in the oral biofilm dental plaque. To adhere to tooth surfaces in dental plaque and to survive, *S. mutans* produces several extracellular proteins or enzymes, including those classified as wall-anchored proteins. Five genes encoding wall-anchored proteins have been characterized in *S. mutans* up to now: *pac*, *fruA*, *dexA*, *gbpC*, and *wapA* (Ferretti *et al.*, 1989; Okahashi *et al.*, 1989; Burne and Penders, 1992; Igarashi *et al.*, 1995; Sato *et al.*, 1997). This group of proteins is exported, but also is tethered to the peptidoglycan of the cell wall by the extracellular sortase enzyme (Cossart and Jonquieres, 2000). Furthermore, these proteins are characterized by a common structure that has been well-documented (Navarre and Schneewind, 1999).

Recently, we identified a gene tentatively designated *cnm* (DDBJ Acc. #AB102689) involved in the cold-agglutination phenotype of *S. mutans* strain Z1 following *in vitro* random mutagenesis mediated by the *HimarI* minitransposon introduced into the chromosomal DNA of the strain (Sato *et al.*, 2004). This gene encoded a new member of wall-anchored proteins, which was highly homologous to a group of collagen-adhesin proteins from *Staphylococcus aureus* (Patti *et al.*, 1992) as well as other bacteria (Rich *et al.*, 1999; Lannergard *et al.*, 2003; Nallapareddy *et al.*, 2003). Some strains of *S. mutans* were reported to recognize and bind collagen (Liu *et al.*, 1990; Switalski *et al.*, 1993). Although the extracellular protein antigen I/II of *S. mutans* was reported to be involved in the binding of cells to extracellular matrix proteins including collagen (Love *et al.*, 2000; Beg *et al.*, 2002), Cnm protein may be a strain-specific collagen-binding molecule in this species. In the present communication, we describe the characterization of the collagen-binding activities of the protein and whole cells of the *S. mutans* strains.

## MATERIALS & METHODS

### Bacterial Strains

The *S. mutans* strain used for random mutagenesis was Z1, which is one of the previously isolated strains from Tokyo Dental College. Its mutant 05A02, in which the *cnm* gene was interrupted by minitransposon insertion, was isolated following application of an *in vitro* random mutagenesis strategy described in detail in our recent report (Sato *et al.*, 2004). Strain Z1 was detected as a colony morphologically specific for *S. mutans* on the Mitis-Salivarius-Bacitracin agar plate and exhibited an *S. mutans*-specific biotype as reported by Shklair and Keene (1974). The nucleotide sequence of the 16S rRNA gene from strain Z1 indicated that this strain belongs to an *S. mutans* species (Bentley *et al.*, 1991).

According to a PCR method recently developed to distinguish serotypes of

*S. mutans* (Shibata *et al.*, 2003), strain Z1 was determined to be serotype f. Other *S. mutans* strains used are ATCC10449, PS14, Ingbritt, GS-5, MT703, MT8148, V403, UA101, UA159, NG8, LM7, PMZ175, and 109c. Streptococci were maintained and cultured in Todd-Hewitt (TH) broth/agar plates, and kanamycin (Km) was added at 500 µg/mL in the media where indicated. *Escherichia coli* strain TOP10, obtained from a commercial supplier (Invitrogen, Carlsbad, CA, USA), was used as a host for plasmid pBAD/His and its derivatives, and strain DH5α was routinely used for standard procedures of DNA manipulation (Noran, 1989), except as indicated.

**Nucleotide Sequence Analysis**

We used BLAST programs to search the *S. mutans* genome Database at the University of Oklahoma's Advanced Center for Genome Technology (<http://www.genome.ou.edu/smutans.html>) and the International DNA databases (EMBL, GenBank, and DDBJ) for similar amino acid sequences. Sequence analysis and multiple alignment were carried out with the DNASIS-Mac program (Hitachi Software Engineering, Yokohama, Japan).

**Cloning and Expression of the Collagen-binding Domain of the Cnm Protein**

A gene fragment corresponding to the predicted collagen-binding domain of Cnm protein was amplified by PCR with the primers 120-kDa FwFu (5'-ATCTGCAGTGATGTCAGCAGTAACATTTCA-3') and DSHpa3R (5'-CTGTAGTAGTGGTTGTTCTTCCGT-3') and ligated inframe to the 5' histidine-tag region (*Pst*I site) of an expression vector, pBAD/HisA (Invitrogen). Following transformation with *E. coli* strain TOP10, the resulting clones were analyzed as described previously (Noran, 1989). One of these clones, ZAXF, was used for collagen-binding assays along with strain TOP10 harboring the vector pBAD/HisA (strain pBAD) and also harboring plasmid pSBP6 expressing another histidine-tagged protein [strain SBP6 (Sato *et al.*, 2002a)] as negative controls. Cells of these strains grown with or without 2 x 10<sup>-3</sup>% arabinose as an inducer were collected, washed, and subjected to 6 cycles of ultrasonication as described previously (Sato *et al.*, 2002b) to obtain crude cell-free extracts for the collagen-binding assays. Induction of the histidine-tagged proteins was confirmed with SDS-PAGE and CBB staining before the assays.

**Binding of Recombinant Cnm to ECM Proteins**

An ELISA used to analyze the binding ability of recombinant protein to immobilized ECM proteins was carried out according to the procedure described recently (Nallapareddy *et al.*, 2003), with slight modifications. Briefly, ELISA plates (Code 3801-096, Asahi Techno Glass Corporation, Funabashi city, Japan) were coated with 1 µg of ECM proteins or bovine serum



**Figure 1.** Alignment of Cnm CBD with CBDs from previously identified collagen-binding adhesins. All 5 sequences were numbered from the initiation codon of the precursor proteins. The putative CBD identified from the Cnm sequence was aligned with CBDs from *S. aureus*, *E. faecium*, *S. equi*, and *E. faecalis*, with the use of the DNASIS-Mac program. Identical amino acid residues are indicated as letters on a gray background.

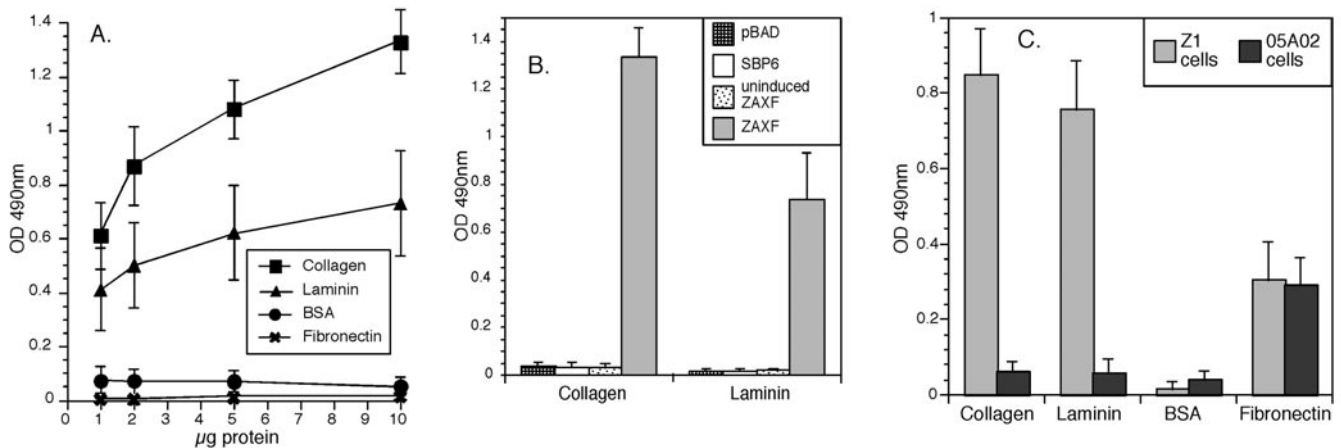
albumin (BSA) in 100 mL of PBS (50 mM potassium phosphate, pH 7.2; 150 mM NaCl) and allowed to incubate overnight at 4°C. After the plates were washed with PBST (PBS with 0.01% Tween 20) and blocked with 5% BSA, various amounts of cell-free extracts (1-10 µg proteins in 20 µL of PBS with 0.1% BSA) were added to the wells and incubated for 1.5 hrs at 37°C. Bound proteins were detected by Anti-His HRP Conjugates (Qiagen) antibody.

**Binding of Wild-type and Mutant Whole Cells of S. mutans to ECM Proteins**

We used an ELISA to evaluate the binding abilities of whole cells of wild-type strains, including Z1 and mutant 05A02, to immobilized ECM proteins according to the procedure described previously (Ruhl *et al.*, 1996), with a slight modification. ECM proteins were coated, washed, and blocked as described above. The whole cells were harvested from overnight cultures, washed 3x with PBS, and adjusted to a turbidity of 1.0 with a spectrophotometer (Ubest35, JASCO Corporation, Tokyo, Japan), equivalent to approximately 1 x 10<sup>9</sup> cfu/mL. A 0.9-mL portion of the cells was biotin-labeled by being mixed with a 0.1-mL PBS solution containing 0.1 mg of NHS-LC-Biotin (Pierce, Rockford, IL, USA). Labeled cells were washed in the inner tubes of Ultrafree-CL centrifugal filter devices (0.22 µm, Millipore Corporation, Bedford, MA, USA) by 3 rounds of filtration and resuspension of cells. Approximately 2 x 10<sup>8</sup> cells were used for the binding assay and incubated for 1 hr at 37°C. Bound cells were detected by Streptavidin-HRP Conjugates (Amersham Biosciences, Piscataway, NJ, USA).

**Southern Hybridization Analysis**

The restriction enzyme (*Hind*III)-digested chromosomal DNA fragments from strain Z1, reference strains including strain UA159, and natural isolates were analyzed by use of the ECL direct nucleic-acid-labeling and detection system (Amersham Co.



**Figure 2.** Binding of recombinant putative CBD of Cnm protein to immobilized ECM proteins. **(A)** Strain ZAXF was grown in the presence of  $2 \times 10^{-3}\%$  arabinose as an inducer, and the crude cell-free extract was prepared as described in the text. Binding of recombinant Cnm protein to immobilized ECM proteins, collagen type I, fibronectin, and laminin was indicated as a function of protein concentration (1–10  $\mu\text{g}$  in 20  $\mu\text{L}$  of PBS with 0.1% BSA) of the extracts applied to the wells. BSA was used as a negative control. **(B)** The extract contained 10  $\mu\text{g}$  protein from strain ZAXF cells grown in the absence of arabinose (uninduced ZAXF), and those from strains pBAD (as a negative control) and SBP6 (a strain expressing another histidine-tagged protein as a negative control) cells grown in the presence of  $2 \times 10^{-3}\%$  arabinose were also used as controls for binding of recombinant Cnm protein to collagen type I and laminin. **(C)** Biotin-labeled strains Z1 and 05A02 whole cells were examined for binding to the ECM proteins as described in the text.

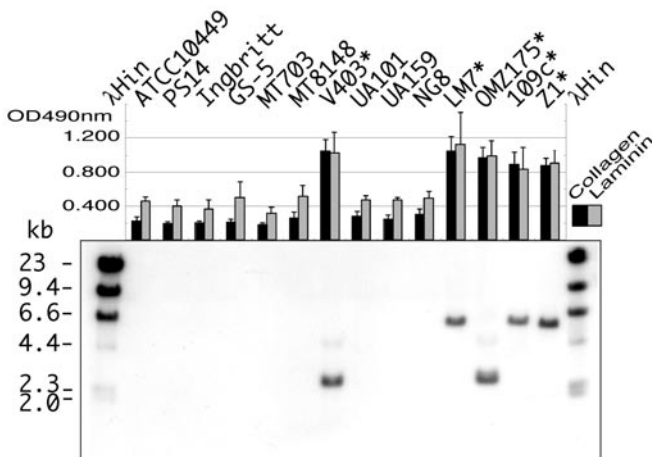
We measured relative binding by monitoring absorbance at 490 nm following the peroxidase reaction for 3 min in the recombinant assays and for 4 min in whole-cell assays with *o*-phenylenediamine, and  $\text{H}_2\text{O}_2$  was terminated with the addition of 2 M  $\text{H}_2\text{SO}_4$ . All OD490 nm values were corrected for the responses of peroxidase activities with the respective ECM proteins. Data points represent the means of OD490 nm values  $\pm$  standard deviation from more than 3 independent experiments.

LTD., Tokyo, Japan) as described previously (Sato *et al.*, 1997).

## RESULTS

### Characteristics of the Cnm Protein Sequence

A nucleotide sequence homologous to the *cnm* gene was not



**Figure 3.** Presence of the *cnm* gene in a population of *S. mutans*. *Hind*III-digested chromosomal DNA fragments from reference strains, including strain UA159, and several natural isolates in addition to strain Z1 were analyzed by Southern hybridization under high stringency conditions, with the *cnm* gene fragment as a probe. Asterisked are the *cnm* gene-positive strains. Binding assays of the strains to collagen/laminin were carried out as in Fig. 2. Data points represent the means of OD490 nm values  $\pm$  standard deviation from 4 (collagen) or 3 (laminin) independent experiments.

detected in the UA159 genome database. The DNA databases were then searched for similar proteins with the amino acid sequence deduced from the *cnm* gene. Similar sequences were found in a group of collagen-binding adhesin proteins, which are also wall-anchored proteins, from staphylococci, enterococci, and equine streptococci. The most similar target sequence regions were those corresponding to the collagen-binding domains (CBD) in collagen-binding adhesin precursor proteins (Cna) from *S. aureus* strain FDA 574 (54.8% identity) and MRSA strain MW2. The next most similar sequences were the recently reported CBDs of the *Enterococcus faecium* (Acm, 48.8% identity) and *Streptococcus equi* (Cne, 48.2% identity). The CBD of the *Enterococcus faecalis* (Ace, 31.5% identity) was less similar to that of *S. mutans* Cnm. Multiple alignments of the 5 CBD sequences are presented in Fig. 1.

Another characteristic sequence of the Cnm protein was found in the C-terminal region as a repetitive sequence, which consists of tandem TTTTE(K/A)P, and subsequent 19 TTTTE(A/S/T)P repeats (Fig. 4).

### Collagen-binding Assay

Based on the similarities of the N-terminal amino acid sequence deduced from the *cnm* gene to the CBD in collagen-binding adhesin precursor proteins, a 5' *cnm* gene region corresponding to the mature N-terminal region containing the putative CBD region was amplified by PCR and subcloned into a pBAD/HisA expression vector to overexpress the protein. Since ZAXF protein expression was easily confirmed by CBB staining of SDS polyacrylamide gels, we initially attempted to purify the protein using a commercially available ProBond resin column system

(Invitrogen). However, the protein aggregated immediately after elution under native conditions with imidazol as recommended by the supplier. In addition, the protein obtained by pH elution did not exhibit any binding activity to collagen. Therefore, we used the crude *E. coli* extracts as described in MATERIALS & METHODS. The ZAXF protein bound to immobilized collagen type I in a concentration-dependent manner and also, to a lesser extent, to laminin but not to fibronectin or BSA (Fig. 2A). Protein from ZAXF cells grown in the absence of arabinose (uninduced ZAXF) and that from strains pBAD and SBP6 cells grown in the presence of 2 x 10<sup>-3</sup>% arabinose did not exhibit binding to collagen type I and laminin (Fig. 2B).

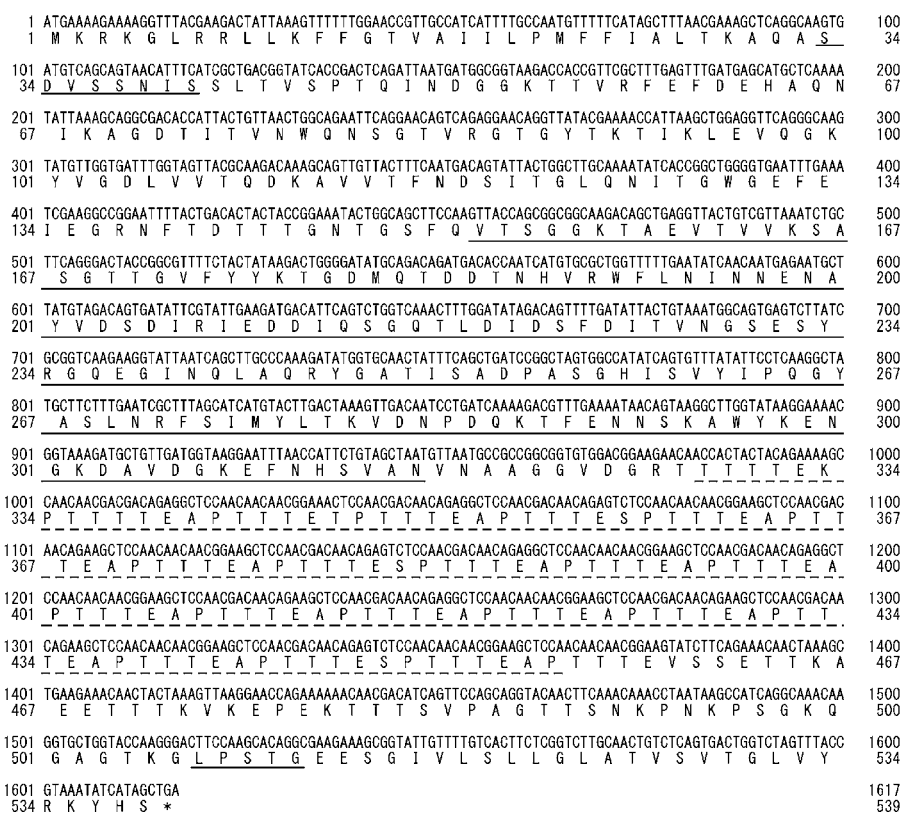
Biotin-labeled *S. mutans* Z1 whole cells bound collagen type I and laminin best and interacted with fibronectin to a lesser extent (Fig. 2C). In contrast, mutant 05A02 cells bound only to fibronectin, with binding activity similar to that of Z1 cells. The binding profiles of both the recombinant and whole-cell assays were comparable.

**Prevalence of the *cnm* Gene among *S. mutans* Strains**

To evaluate the occurrence of the *cnm* gene among different *S. mutans* strains, we carried out Southern blot analysis of *Hind*III-digested chromosomal DNA fragments from laboratory strains and isolates, including UA159 and Z1, using the *cnm* gene fragment as a probe. Five out of the 14 strains examined were *cnm*-positive (Fig. 3) and exhibited cold-agglutination and collagen/laminin-binding activities, while the other 9 strains did not exhibit these phenotypes. Interestingly, 3 of the 5 positive strains were serotype e or f (LM7, OMZ175, and Z1), but no clonality has been observed among these *cnm*-positive strains analyzed by AFLP.

**DISCUSSION**

The Cnm protein exhibited high homology with collagen-binding adhesins from *S. aureus*, *E. faecium*, and *S. equi* (Patti *et al.*, 1992; Lannergard *et al.*, 2003; Nallapareddy *et al.*, 2003). Specifically, the 165-amino-acid sequence from residues 152 to 316 was highly homologous to the CBDs of these collagen-binding adhesins. In addition, several amino acids that are critical for collagen-binding by Cna of *S. aureus* (Patti *et al.*, 1995; Symersky *et al.*, 1997), including those which form the walls of the groove in Cna accepting collagen molecules (Symersky *et al.*, 1997), were conserved in all 5 CBDs. These results suggested that the observed collagen-binding activity of *S. mutans* Cnm protein was highly predictable.



**Figure 4.** The DNA nucleotide and deduced amino acid sequences of the *cnm* gene. (The DDBJ-EMBL-GenBank nucleotide sequence databases accession number is AB102689.) Underlines indicate, respectively, the N-terminal sequence determined from isolated Cnm protein, CBD homologous sequence, putative B repeats domain, and LPXTG motif.

Previously identified collagen-binding adhesin molecules (Patti *et al.*, 1992; Rich *et al.*, 1999; Lannergard *et al.*, 2003; Nallapareddy *et al.*, 2003) contained the B repeat regions following the A domain containing the CBD. However, the number of repeats and the length of the repeating units were dependent on species variation. *S. mutans* Cnm contained 2 seven-residue and 19 six-residue repeating units (Fig. 4) located between the putative CBD and C-terminal wall-associated domain rich in proline and lysine residues. This repeat region of the Cnm protein may correspond to the B domain of the collagen-binding adhesins. Therefore, we conclude that the characteristic domain structure of the Cnm protein, as well as the homology of its putative CBD, was conserved overall.

Specific binding assays with crude *E. coli* extracts containing a recombinant CBD domain (ZAXF protein) revealed that the *S. mutans* Cnm protein is a new member of the collagen-binding adhesin family. It is of interest that the ZAXF protein also exhibited an affinity to laminin. This was compatible not only with the demonstrated relative collagen- and laminin-binding properties of intact cells of Z1 and its *cnm* mutant 05A02, but also with the similar fibronectin-binding abilities retained by both strains. The Southern blot analysis of *S. mutans* strains, with the *cnm* gene fragment as a probe, revealed that 9 out of 14 strains, including strain UA159, do not

harbor this gene. More than a decade ago, two reports (Liu *et al.*, 1990; Switalski *et al.*, 1993) demonstrated that approximately 20 and 25% of *S. mutans* strains tested were able to bind collagen. These ratios were comparable with the present results. Taken together, these results suggested that the collagen-binding of *S. mutans* cells is likely mediated by the strain-specific Cnm protein.

Oral viridans streptococci are pathogens associated with infective endocarditis, and the binding abilities of these organisms to subendothelial matrix proteins including collagen, sialoproteins, fibronectin, and laminin, as well as blood-derived fibrinogen (fibrin), are regarded as potential virulence factors (Sommer *et al.*, 1992; Sciotti *et al.*, 1997; Chia *et al.*, 2000; Beg *et al.*, 2002; Takahashi *et al.*, 2002). Although *S. mutans* was reported to be responsible for 8-18% of total streptococcal endocarditis (Ryd *et al.*, 1996), and extracellular protein antigen I/II was reported to be involved in binding of *S. mutans* cells to extracellular matrix protein (Love *et al.*, 2000; Beg *et al.*, 2002), including collagen, it has often been questioned whether *S. mutans* is a true pathogen in endocarditis. In this respect, strain-specific Cnm protein expression may be an important virulence factor and may provide an answer to this question. Therefore, it will be of interest to compare the percentage of the *cnm* gene-positive strains isolated from infective endocarditis patients with that from healthy people, and also of interest to see whether the *cnm* gene-positive or -negative strains differentially induce experimental endocarditis in a rat-model system.

To our knowledge, this is the first report that demonstrates a collagen-binding adhesin from viridans streptococci in human oral indigenous flora.

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