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Identification and characterization of a collagen-binding protein, Cbm, in *Streptococcus mutans*

R. Nomura[1], K. Nakano[1], S. Naka[1], H. Nemoto[1], K. Masuda[1], J. Lapirattanakul[2], S. Alaluusua[3,4], M. Matsumoto[1], S. Kawabata[5] and T. Ooshima[1]

1 Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan
2 Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand
3 Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland
4 Department of Pediatric and Preventive Dentistry, Institute of Dentistry, University of Helsinki, Helsinki, Finland
5 Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan

Correspondence: Kazuhiko Nakano, Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, 1-8 Yamada-oka, Suita, Osaka 565-0871, Japan Tel.: + 81 6 6879 2963; fax: + 81 6 6879 2965; E-mail: nakano@dent.osaka-u.ac.jp

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SUMMARY

***Streptococcus mutans*, a major pathogen of dental caries, is occasionally isolated from the blood of patients with infective endocarditis. Bacterial attachment of exposed collagen tissue in the impaired endothelium is an important step in the onset of infective endocarditis. In our previous studies, some *S. mutans* strains were shown to possess collagen-binding activities and most of them had an approximately 120-kDa cell-surface collagen-binding protein called Cnm. However, several strains without Cnm proteins show collagen-binding properties. In the present study, another collagen-binding protein, Cbm, was characterized and its coding gene *cbm* was sequenced in these strains. The amino acid alignment in the putative collagen-binding domain of Cbm was shown to have approximately 80% identity and 90% similarity to the comparable region of Cnm. Cbm-deficient isogenic mutant strains constructed by insertional inactivation of the *cbm* gene, lacked collagen-binding properties, which were recovered in the complemented mutant. Analyses of a large number of clinical isolates from Japan, Thailand and Finland revealed that *cbm*-positive strains were present in all of these countries and that *cnm*-positive and *cbm*-positive strains were detected in the oral cavity of approximately 10 and 2% of systemically healthy subjects, respectively. In addition, *cnm*-positive strains were predominantly identified in the serotype *f* group, whereas *cbm*-positive strains were frequently detected in serotype *k*. These results suggest that Cbm as well as Cnm are major cell surface proteins of *S. mutans* associated with binding to type I collagen and predominantly identified in serotype *k* strains.**

INTRODUCTION

Streptococcus mutans is generally considered to be a major pathogen of dental caries, and is also detected in blood, which is associated with bacteremia and infective endocarditis (IE) (Nakano & Ooshima, 2009). The *S. mutans* strains are classified into four serotypes (*c*, *e*, *f* and *k*) based on the chemical composition of the serotype-specific rhamnose-glucose polymers (Hamada & Slade, 1980; Nakano *et al.*, 2004a). Most of the oral strains are reported to be serotype *c* at a prevalence greater than 70%, followed by serotype *e* (approximately 20%), whereas

the distribution frequency for serotypes *f* or *k* was reported to be lower than 5% for each (Hirasawa & Takada, 2003; Shibata *et al.*, 2003; Nakano *et al.*, 2004a,b; Lapirattanakul *et al.*, 2009; Nakano & Ooshima, 2009). Serotype *k* was the most recently designated serotype using non-*c*/*e*/*f* blood isolates from Japanese patients (Nakano *et al.*, 2004a). Also, its distribution has been confirmed in UK, Finland, Thailand and its existence is suspected in Chile (Nakano *et al.*, 2010a).

The complete genome sequences of two serotype *c* *S. mutans* oral strains have been determined, revealing approximately two million nucleotides and leading to a better understanding of the molecular biological properties of these strains (Ajdić *et al.*, 2002; Maruyama *et al.*, 2009). As for major cell surface protein antigens of *S. mutans*, three types of glucosyltransferases and the approximately 190-kDa protein antigen, sometimes designated as PAc (described as antigen I/II and other names), are considered to be important in the development of dental caries (Aoki *et al.*, 1986; Pucci *et al.*, 1987; Hanada & Kuramitsu, 1989; Okahashi *et al.*, 1989). Several surface proteins, such as PAc, have been shown to have a collagen-binding capability (Beg *et al.*, 2002). Recently, an approximately 120-kDa Cnm protein related to the collagen-binding activity of *S. mutans* was characterized (Sato *et al.*, 2004). This protein was reported to consist of a collagen-binding domain, a putative B-repeat domain, and a cell-wall-anchored LPXTG motif. The distribution frequency of the strains with the *cnm* gene among oral isolates has been estimated to be approximately 10–20% and *cnm*-positive strains are known to possess high collagen-binding properties (Sato *et al.*, 2004; Nomura *et al.*, 2009; Nakano *et al.*, 2010b; Lapirattanakul *et al.*, 2011). These strains are predominantly identified in serotypes *f* or *k* strains, even though these are considered to be minor serotypes in the oral cavity (Lapirattanakul *et al.*, 2009; Nomura *et al.*, 2009). Recently, analyses using human coronary artery endothelial cells showed that Cnm is required for *S. mutans* invasion of endothelial cells, indicating its possible contribution to cardiovascular infections and pathology (Abranches *et al.*, 2011). In addition, Cnm of *S. mutans* was also shown to be involved in the deterioration of cerebral hemorrhage (Nakano *et al.*, 2011).

In our previous study, we identified several clinical strains of *S. mutans*, predominantly in the serotype *k* group, which did not possess *cnm* genes but exhibited collagen-binding activity (Nomura *et al.*, 2009; Nakano *et al.*, 2010b). Hence, we speculate that other unknown proteins with collagen-binding properties could exist in these serotype *k* strains. In the present study, a collagen-binding protein of *S. mutans* was identified and named Cbm, (collagen-binding protein of *S. mutans*) and its coding gene *cbm*, which may or may not be related to *cnm*, was characterized. In addition, the distribution of the strains with the *cbm* gene was investigated using clinical isolates from three different countries.

METHODS

S. mutans strains

A total of 580 *S. mutans* isolated from the oral cavities of 320 Japanese, 150 Thai and 110 Finnish healthy subjects were analysed (Nakano *et al.*, 2004a, 2007a; Lapirattanakul *et al.*, 2009; Lapirattanakul *et al.*, 2011). The Japanese subjects comprised 210 children (101 boys and 109 girls; 2–16 years of age; median 6 years old) and 110 adults (48 men and 62 women; 20–82 years of age; median 56 years old). In addition, two blood isolates from Japanese patients with bacteremia (strain TW295) and IE (strain TW871), one oral isolate from a Japanese patient with an aortic aneurysm (strain OR22P1), as well as two oral isolates from a healthy Japanese child (strain NN2193-1) and an adult (strain NN2323M-1) were also included (Fujiwara *et al.*, 2001; Nakano *et al.*, 2007a, 2008). All strains were confirmed to be *S. mutans* based on biochemical properties and observation of a rough colony morphology on Mitis-salivarius (MS) agar (Difco Laboratories, Detroit, MI) plates containing bacitracin (0.2 U ml^{-1}; Sigma Chemical Co., St Louis, MO) as well as 15% (weight/volume) sucrose (MSB agar). In addition, serotype determination was carried out using immunological and polymerase chain reaction (PCR) methods, as described previously (Shibata *et al.*, 2003; Nakano *et al.*, 2004b).

Identification of the *cbm* gene encoding Cbm

First, the primer set (cnm-F1/cnm-R1) was designed corresponding to the interior of the *cnm* gene and other sets (cnm-F2/cnm-R2 and cnm-F3/cnm-R3) were also designed from the *cnm* flanking regions

Table 1 Polymerase chain reaction primer sets used in the present study

Purpose	Name	Sequence (5′–3′)[1]
cbm sequence	cnm-F1	CAT TTT GCC AAT GTT TTT CA
	cnm-R1	CCT GTG CTT GGA AG
	cnm-F2	AAT CTG GGC AAT ATC ACA CA
	cnm-R2	TCA TCA AAC TCA AAG CGA AC
	cnm-F3	AAC AAA GGC TGA AGA AAC GA
	cnm-R3	TTC TTC ATT ATA AAA AGC GA
	cbm-1F	GAC AAA CTA ATG AAA TCT AA
	cbm-1R	TCA TCA GGA ACC AGC GCA CA
	cbm-2F	AGC TGA AGT TAG TGT TGT AA
	cbm-2R	ATG CCG CCG GCA GCA TTA AC
	cbm-3F	CAA TAG TAA AGC TTG GTA CA
	cbm-3R	GCA AAA ACT GTT GTC CCT GC
cbm expression	cbm-EF	AGC TGA AGT TAG TGT TGT AAA ACC TGC TTC
Southern hybridization, *cbm* detection	cbm-ER	TAG GAT CAT CAA CCT TAG TCA AGT ACA CGA
Recombinant Cbm generation	cbm-RF	CCC GGG AGA AAG GAA TTC AAA ATG AAA AGA
	cbm-RR	TCC TGT CTC GAG TCA ACA TCA GCT ATG
Recombinant Cnm generation	cnm-RF	CGG GAA GGA TCC AAA AAT ATG AAA AGA
	cnm-RR	GTC GCT GCA GCT TTC ATC CTG TTT TTA A
cbm-mutant strain generation	EmrAgeI-F	CGC CGG ACC GGT TAC ATG AAC AAA AAT
	EmrAgeI-R	GCG ACC GGT AGA ATT ATT TCC TCC CG
	KmrAgeI-F	TGA CCG GTA AGA TTA TAC CGA GGT A
	KmrAgeI-R	GTA CTA AAC CGG TTC ATC CAG TAA A
cnm detection	cnm-DF	TGG AGG TTC AGG GCA AGT ATG TTG GTG ATT
	cnm-DR	GTC TTT TGA TCA GGA TTG TCA ACT TTA GTC

[1]Underlining indicates the restriction sites.

based on the sequence of strain TW871 (GenBank accession number AB469914) (Table 1 and Fig. 1A). Strain YT1 was selected as a representative strain, which showed collagen-binding properties but demonstrated negative reactions by Western blots with anti-Cnm as well as Southern blots with a *cnm* probe. The PCR was carried out in this *cnm*-negative strain YT1 with collagen-binding properties with primer sets cnm-F1/cnm-R1, cnm-F2/cnm-R2 and cnm-F3/cnm-R3 using TaKaRa Ex Taq (Takara Bio. Inc., Otsu, Shiga, Japan). The amplified fragment was then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and the nucleotide sequence was determined using a dye-terminator reaction with a DNA sequencing system (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) and BigDye terminator cycle sequencing kit. The sequences obtained were combined using GENE WORKS software (IntelliGenetics, Mountain View, CA), which resulted in the specification of a 1704 bp open reading frame for a gene (named *cbm*) presumably associated with collagen-binding properties as well as its adjacent regions. Next, the entire nucleotide alignment of the obtained sequence was confirmed using the three fragments amplified by PCR using the primer sets (cbm-1F/cbm-1R, cbm-2F/cbm-2R and cbm-3F/cbm-3R) designed on the basis of the obtained nucleotide alignments (Table 1 and Fig. 1A). The nucleotide sequence of *cbm* was determined for 10 serotype *k* as well as one serotype *c*, three serotype *e* and one serotype *f* strains (GenBank accession numbers: AB610578–AB610592) (Tables 2 and 3). A homology search of the nucleotide alignment of *cbm* and the putative amino acid sequence of its product were performed using GenBank, EMBL, and DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server (http:/http://www.ncbi.nlm.nih.gov/BLAST/).

Evaluation of *cbm* expression in clinical isolates

The reverse transcript–PCR (RT-PCR) method was performed to confirm the transcription of the *cbm* genes by the method described previously (Nomura *et al.*, 2005). Initially, total RNA was prepared from

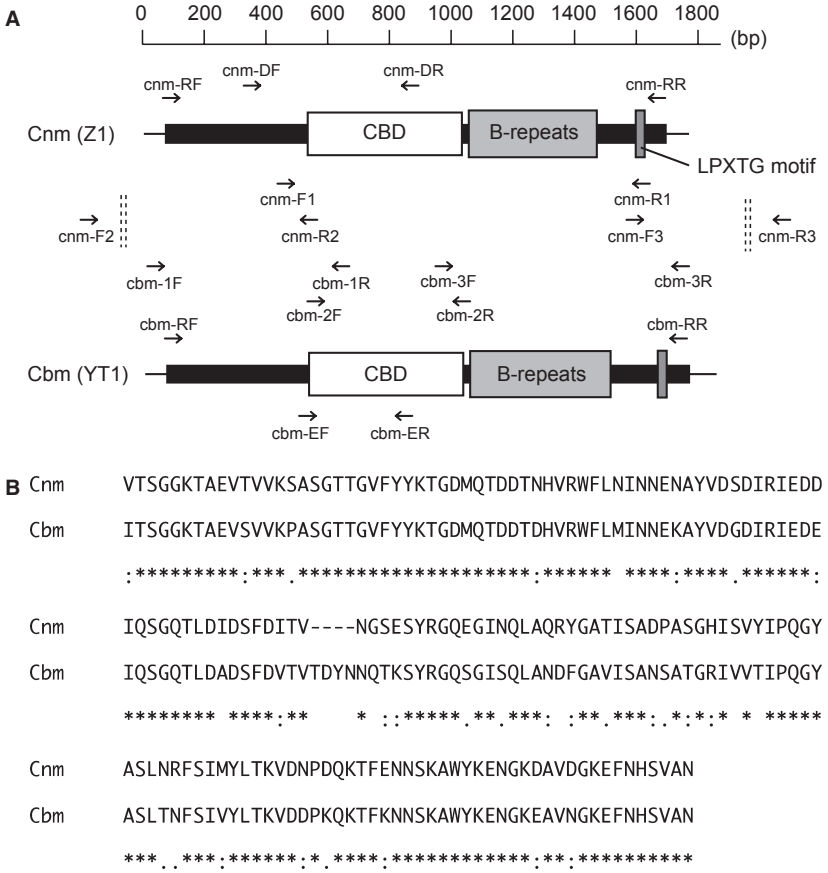

Figure 1 Illustration of the putative structure of Cnm and Cbm in *Streptococcus mutans*. (A) Putative structure of Cbm (strain YT1) as compared with that of Cnm (strain Z1). Horizontal lines indicate the entire length of the *cnm* and *cbm* genes. Filled rectangles show the open reading frames. CBD, Collagen-binding domain. Arrows indicate the primer positions, designed based on the nucleotide alignment of the *cnm* of Z1 or *cbm* of YT1. (B) Comparison of the putative amino acid alignment for the CBD of Cbm (strain YT1) with that of Cnm (Strain Z1). Asterisks indicate identical amino acid residues between the two sequences. Single and double dots indicate weakly and highly similar amino acid residues, respectively. Dashes indicate the gaps when multiple alignments were performed.

the test strains using a FastPrep® Cell Disrupter (Model FP100A; Q-Bio gene, Carlsbad, CA) in combination with a Fast RNA® Pro Blue kit (Q-Bio gene), according to the manufacturer's instructions. RQ1 RNase-Free DNase (Promega) and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) were used to amplify cDNA synthesized from mRNA. The primer set cbm-EF and cbm-ER (Table 1 and Fig. 1A) was designed to detect *cbm* transcription. Successive PCR assays were performed using TaKaRa ExTaq with primer set cbm-EF and cbm-ER under the following conditions: an initial denaturation at 95°C for 4 min and then 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 7 min. Genomic DNA from each strain was used as a positive control and RNA samples without reverse transcriptase served as negative controls.

Generation of antiserum against Cbm

Recombinant Cbm and anti-Cbm serum samples were generated as reported previously (Matsumoto-Nakano *et al.*, 2007). The coding regions of *cbm* in strain SA31 (GenBank accession number AB610585) were amplified using genomic DNA by PCR using TaKaRa Ex Taq (Takara Bio) with primer sets constructed based on sequences that added a restriction enzyme site at the 5′ and 3′ ends (cbm-RF and cbm-RR) (Table 1 and Fig. 1A). The amplified fragments of *cbm* were subcloned into the GST fusion expression vector pET-42a(+) (Novagen, Darmstadt,

Table 2 Serotype *k Streptococcus mutans* strains analysed in the present study

Strains	Features	Isolated countries	Collagen-binding activities[3]	Identification of the gene		GenBank accession numbers	References
				cnm	*cbm*		
TW295	Blood isolate from a patient with bacteremia after tooth extraction	Japan	+	+	–	AB469913	Fujiwara *et al.* (2001)
TW871	Blood isolate from a patient with infective endocarditis	Japan	+	+	–	AB469914	Fujiwara *et al.* (2001)
LJ23	Oral isolate from a healthy subject[2]	Japan	+	+	–	AB465261	Nakano *et al.* (2007a)
OR22P1	Oral isolate from a patient with aortic aneurysm	Japan	+	+	–	AB600185	Nakano *et al.* (2007a)
TLJ60-1	Oral isolate from a healthy subject[2]	Thailand	+	+	–	AB600186	Lapirattanakul *et al.*, (2011)
SA53	Oral isolate from a healthy subject[2]	Finland	+	+	–	AB465299	Nakano *et al.* (2007a)
YT1	Oral isolate from a healthy subject[2]	Japan	+	–	+	AB610578	Nakano *et al.* (2004a)
AT1[1]	Oral isolate from a healthy subject[2]	Japan	+	–	+	AB610579	Nakano *et al.* (2004a)
NN2193-1[1]	Oral isolate from a healthy subject	Japan	+	–	+	AB610580	Nakano *et al.* (2007a)
NN2323M-1	Oral isolate from a healthy subject	Japan	+	–	+	AB610581	Nakano *et al.* (2007a)
SN3027	Oral isolate from a healthy subject[2]	Japan	+	–	+	AB610582	This study
TLJ11-2[1]	Oral isolate from a healthy subject	Thailand	+	–	+	AB610583	Lapirattanakul *et al.* (2009)
TLJ85-4[1]	Oral isolate from a healthy subject	Thailand	+	–	+	AB610584	Lapirattanakul *et al.*, (2011)
SA31[1]	Oral isolate from a healthy subject[2]	Finland	+	–	+	AB610585	Nakano *et al.* (2007a)
SA72	Oral isolate from a healthy subject[2]	Finland	+	–	+	AB610586	Nakano *et al.* (2007a)
SA98[1]	Oral isolate from a healthy subject[2]	Finland	+	–	+	AB610587	This study
FT1	Oral isolate from a healthy subject[2]	Japan	–	–	–	N/A	Nakano *et al.* (2004a)
TLJ26-1	Oral isolate from a healthy subject[2]	Thailand	–	–	–	N/A	Lapirattanakul *et al.* (2009)
TLJ106-1	Oral isolate from a healthy subject[2]	Thailand	–	–	–	N/A	Lapirattanakul *et al.*, (2011)

[1]Strains with construction of *cbm*-inactivated mutants. In addition, a complemented mutant was generated using SA31.
[2]Strains analysed for serotype distribution in Table 5.
[3]'+' and '–' indicate positive and negative binding to type I collagen *in vitro*.
N/A, not applicable.

Table 3 *Streptococcus mutans* strains other than serotype *k* with the *cbm* gene

Strains[1]	Features	Isolated countries	GenBank accession numbers	References
NN2094[2] (*e*)	Oral isolate from a healthy subject	Japan	AB610588	Nakano *et al.* (2007a)
TLJ9-1 (*f*)	Oral isolate from a healthy subject	Thailand	AB610589	Lapirattanakul *et al.* (2009)
SA80 (*c*)	Oral isolate from a healthy subject	Finland	AB610590	This study
SA121 (*e*)	Oral isolate from a healthy subject	Finland	AB610591	This study
SA129 (*e*)	Oral isolate from a healthy subject	Finland	AB610592	This study

[1]Parentheses indicate the serotype of each strain.
[2]Strain with constructed *cbm*-inactivated mutants.

Germany), with the resultant plasmid pRN110 transformed into *Escherichia coli* BL21(DE3). The *E. coli* BL21(DE3) transformants carrying pRN110 were grown in Luria–Bertani broth containing kanamycin (30 μg ml^{-1}) at 37°C to the mid-exponential phase. Isopropylthio-β-D-galactoside (Wako Chemical Industries, Osaka, Japan) was then added to produce a final concentration of 1.0 mM, and the cultures were incubated for an additional 3 h to induce GST-Cbm protein synthesis after which the cells were harvested by centrifugation. Pelleted cells were suspended in 10 mM phosphate buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and ultrasonicated on ice. Supernatants were obtained by centrifugation and purified using a glutathione Sepharose™ 4B column (GE Healthcare, Uppsala, Swe-

den). After the GST fusion proteins were treated with PreScission™ Protease (GE Healthcare) at 4°C, recombinant Cbm (rCbm) was purified separately using the same column. The purified rCbm samples were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Antisera against Cbm was prepared by repeated intramuscular injections of rabbits (New Zealand white rabbits; 1–1.5 kg body weight, Oriental Yeast Co. Ltd., Tokyo, Japan) with purified rCbm emulsified with a block copolymer adjuvant (Titer-Max Gold; CytRx Co., Atlanta, GA). The antibody titer of each antiserum sample was then confirmed by Western blot analysis using rCbm. Western blotting of Cbm was then performed using the tested whole bacterial cells and those obtained after 8 M urea treatment as follows. The samples were incubated with 8 M urea at 25°C for 1 h, and saturated with ammonium sulfate, and the precipitate was dialysed for 2 days against 10 mM sodium phosphate buffer (pH 6.0), and was used as a sample.

Recombinant Cnm was generated by amplification of the coding regions of *cnm* from strain TW871 (GenBank accession number AB469914) using the primer set constructed based on sequences that added restriction enzyme sites at the 5′ and 3′ ends (cnm-RF and cnm-RR) (Table 1 and Fig. 1A). The amplified fragments of *cnm* were subcloned into the expression vector pET-42a(+) and the resultant plasmid, pRN210, was transformed into *E. coli* BL21 (DE3). The *E. coli* BL21 (DE3) transformants carrying pRN210 were grown in Luria–Bertani broth containing kanamycin (30 μg ml^{-1}) at 37°C to the mid-exponential phase.

Construction of *cbm*-inactivated and complemented mutants

The *cbm* gene of SA98 was amplified by PCR using primer sets cnm-F1 and cnm-R1 designed based upon the *cbm* sequence of SA98 (Table 1 and Fig. 1A) and ligated into a pGEM-T Easy Vector (Promega) to generate pRN111. The plasmid was then digested with *Age*I for linearization at a unique site and ligated to an *erm* cassette amplified from recombinant plasmid pKN100 (Nakano *et al.*, 2004a) carrying fragments of the erythromycin resistance gene (*erm*) from pVA838 (Macrina *et al.*, 1983) with the primer sets EmrAgeI-F and EmrAgeI-R (Table 1) to yield pRN112. After linearization at the unique *Sac*I restriction site, the plasmid pRN112 was introduced into strain SA98 by transformation to allow allelic exchange using the method described by Tobian & Macrina (1982). The transformants were screened on MS agar plates containing erythromycin (10 μg ml^{-1}). Appropriate insertional inactivation in the mutant strain SA98CBD was confirmed by Southern hybridization with the *cbm* gene amplified by PCR with the primer sets cbm-EF and cbm-ER (Table 1 and Fig. 1A), as well as Western blotting of whole cells of the mutant strains with Cbm-specific rabbit antiserum. The *cbm*-inactivated mutant strains were constructed in the five other serotype *k* strains (AT1, NN2193-1, TLJ11-2, TLJ85-4 and SA31) as well as one serotype *e* strain (NN2094) (Tables 2 and 3).

Next, a complemented mutant of SA31 (strain SA31Comp) was generated as follows. First, the isogenic mutant strain (SA31CBD2) was constructed by insertional inactivation of *cbm* of SA31 using a kanamycin-resistant gene (*aphA*; Caillaud *et al.*, 1987) cassette by the method described previously (Nakano *et al.*, 2002). A shuttle vector plasmid was constructed to express the *cbm* sequence under the control of the inducible TetO/TetR promoter. The entire length of *cbm* was amplified using primer cbm-1F and cbm-3R (Table 1), which was digested with *Sma*I at the multicloning site and was ligated into pTetE (Wang & Kuramitsu, 2005), resulting in generation of shuttle vector plasmid pRN121, which was introduced into *E. coli* DH5α (Nippon Gene, Tokyo, Japan) by transformation. The pRN121 extracted from *E. coli* was then introduced into SA31CBD2 using an electroporation system (Gene Pulser Xcell, Bio-Rad, Hercules, CA). The transformants were screened on MS agar plates containing kanamycin (500 μg ml^{-1}) and erythromycin (10 μg ml^{-1}) to specify the complemented mutant SA31Comp, which was also confirmed by PCR for the appropriate presence of pRN121 in SA31Comp.

Evaluation of collagen-binding activities

The collagen-binding properties of *S. mutans* strains were evaluated according to the method described by Waterhouse & Russell (2006), with some modifications as described previously (Nomura *et al.*, 2009). Type I collagen (type I collagen in 0.25 M acetic acid; Sigma: 2 mg per well) was coated onto 96-well tissue culture plates (Beckton Dickinson, Franklin

Lakes, NJ) and incubated overnight at 4°C. The plates were then washed three times with phosphate-buffered saline (PBS) and blocked for 1.5 h with 5% bovine serum albumin in PBS at 37°C. Next, the wells were washed again with PBS containing 0.01% Tween-20. Cells from overnight cultures of *S. mutans* grown in brain–heart infusion broth (Difco) were collected by centrifugation and the bacterial numbers were diluted with PBS and added to the wells (10^{10} colony-forming units per well). After 3 h incubation at 37°C, adherent cells were washed three times with PBS and fixed with 200 μl 25% formaldehyde at room temperature for 30 min. After another three washes with PBS, the adherent cells were stained with 200 μl 0.05% crystal violet (Wako) in water for 1 min, washed three times with PBS, and then the dye was dissolved by adding 7% acetic acid (200 μl) before determining the optical density at 595 nm. The results for each strain are expressed as a percentage relative to the binding ability of strain TW871, which was defined as 100%. Data are expressed as the mean ± standard deviation of triplicate experiments.

Development of a simple method for identification of *cbm*-positive strains

Based on the nucleotide sequence of *cbm* in 15 strains examined in the present study, a PCR primer set (cbm-EF and cbm-ER) specific for *cbm* was constructed (Table 1 and Fig. 1A). In addition, a *cnm*-specific primer set (cnm-DF and cnm-DR) was also constructed (Table 1 and Fig. 1A) based on the *cnm* sequence determined in the previous studies (GenBank accession numbers: AB102689, AB465259–AB465305, AB469913–AB469914 and AB50097–AB500102). The nucleotide alignment of each primer was confirmed as specific using GenBank, EMBL and DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server. In addition, the specificities of the prospective primers were tested by the program AMPLIFY (Engels, 1993). The estimated amplification size of the PCR products for *cbm*-specific and *cnm*-specific sets of primers were 393 and 579 bp, respectively. PCR amplification was performed in a total volume of 20 μl with 1 μl of template solution and TaKaRa Ex Taq (Takara Bio). The PCR amplification reaction was performed with the following cycling parameters: an initial denaturation at 95°C for 4 min and then 30 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR products were subjected to electrophoresis in a 1.5% agarose gel-Tris-acetate-EDTA buffer. The gel was stained with 0.5 μg ethidium bromide per ml and photographed under UV illumination. A 100-bp DNA ladder (New England BioLabs, Beverly, MA) was used as the molecular size standard. The detection limit for the multiplex PCR detection system for *cnm* and *cbm* was determined by using known numbers of bacterial cells (strains TW295 and YT1) diluted in sterile distilled water. In addition, the sensitivity of the PCR assay was determined by using multiple *cnm*-positive and *cbm*-positive strains.

Prevalence of *cbm*-positive strains in saliva specimens from healthy patients

The study procedures were approved by the Ethics Committee of Osaka University Graduate School of Dentistry and informed consent was obtained from each subject. The saliva specimens were collected from a total of 119 subjects aged 3–20 years in the Department of Pediatric Dentistry, Osaka University Dental Hospital from March to August 2010. Following mouth-washing, expectorated whole saliva was collected from each subject in a sterile plastic tube and immediately placed on ice. The bacterial DNA was extracted by a method described previously (Nomura *et al.*, 2011). Briefly, bacterial cells were collected in a micro-centrifuge tube and incubated with *N*-acetylmuramidase SG (Seikagaku Corp., Tokyo, Japan) and lysozyme (Wako). Genomic DNA was then extracted using a Gentra Puregene Yeast/Bact. Kit B (Qiagen, Tokyo, Japan), according to the manufacturer's instructions.

The PCR was performed using an *S. mutans*-specific primer set by the method described previously (forward; 5′-GGC ACC ACA ACA TTG GGA AGC TCA GTT-3′, reverse; 5′-GGA ATG GCC GCT AAG TCA ACA GGA T-3′) (Hoshino *et al.*, 2004). The PCR was performed using TaKaRa Ex Taq (Takara Bio) involving 30 cycles of a denaturing step at 98°C for 10 s, and a primer-annealing and extension step at 70°C for 1 min. The samples with positive reactions for *S. mutans* were then subjected to the PCR method to detect *cbm*-positive or *cnm*-positive strains by the method developed in the present study.

Evaluation of the adhesion properties to human umbilical vein endothelial cells (HUVEC)

The adhesion properties of *S. mutans* cells to HUVEC were evaluated by the method described previously (Nakano *et al.*, 2010a). Briefly, approximately 1×10^5 HUVEC were seeded in parallel wells of 24-well tissue culture plates (Costar®, Corning, NY). Before infection, the wells were washed three times with PBS and antibiotic-free medium was added. The HUVEC were infected by the addition of 1×10^7 colony-forming units of *S. mutans* cells to the wells. After 90 min of aerobic incubation, the medium was removed and the infected cells were washed three times with PBS. To test for adherence, 1.0 ml sterile distilled water was added to disrupt the cells. Dilutions of cell lysates infected with *S. mutans* were plated onto MSB agar and cultured at 37°C for 48 h under anaerobic conditions. The numbers of the cells adhering to the tissue culture plates (Ntcp) were also measured using the wells incubated with antibiotic-free medium but without infection. The adherence values were determined as (resuspended cells–Ntcp)/infected cells. Data are expressed as the mean ± standard deviation of triplicate experiments.

Statistical analyses

Statistical analyses were carried out using the computational software packages STATVIEW 5.0 (SAS Institute Inc., Cary, NC) and PRISM 4 (GraphPad Software Inc., La Jolla, CA). Biological properties of two groups were compared using Student's *t*-test. Fisher's exact probability test was carried out for analyses of the frequency of serotype distribution. *P*-values <0.05 were considered to be significant.

RESULTS

Molecular characterization of the *cbm* gene

The open reading frame of *cbm* was identified using the sequence of the collagen-binding domain of *cnm*. The *cbm* gene of YT1 consists of 1704 bp encoding 568 amino acids and contains putative regions for a CBD, B-repeats and an LPXTG motif similar to Cnm (Fig. 1A). Determination of the *cbm* sequence in 14 other strains showed that the CBD and LPXTG motifs in the Cbm of all strains were highly conserved, whereas the lengths of the B-repeats varied in each strain, which contained tandem TTTT(AE/TEI)P and subsequent TTTTE(GS/AS/TP/TS) regions (from 16 to 21 repeats). BLAST analyses revealed that the putative amino acid sequence of the CBD of Cbm was highly homologous to that of Cnm of *S. mutans* with 78% identity (Fig. 1B). On the other hand, Table 4 lists the collagen-binding proteins with high homology to the amino acid sequence of the putative Cbm. The protein with the highest homology is the Cnm protein of *S. mutans*, followed by Cna of *Staphylococcus aureus* and Acm of *Enterococcus faecium*. In addition, RT-PCR analyses revealed that mRNA expression of *cbm* was apparent in all *cbm*-positive strains, and Western blotting of Cbm also demonstrated that positive bands corresponding to Cbm were also detected in all *cbm*-positive strains.

Generation of Cbm-defective isogenic mutant strains

Cbm-defective isogenic mutant strains were constructed from seven strains with *cbm* genes (Tables 2 and 3). Southern hybridization analyses showed the

Table 4 Identification of the proteins with high homology to the putative amino acid sequence of the collagen-binding domain of Cbm using BLAST

Proteins	Species	GenBank accession numbers	BLAST results		References
			Identity (%)	Similarity (%)	
Cnm	*Streptococcus mutans*	AB102689	132/169 (78)	148/169 (87)	Sato *et al.* (2004)
Cna	*Staphylococcus aureus*	M81736	91/170 (53)	116/170 (68)	Patti *et al.* (1992)
Acm	*Enterococcus faecium*	AY135217	82/171 (47)	112/171 (65)	Nallapareddy *et al.* (2003)
Cne	*Streptococcus equi*	AY193773	79/168 (47)	108/168 (64)	Lannergard *et al.* (2003)
Acb	*Streptococcus gallolyticus*	GQ497722	75/170 (44)	101/170 (59)	Sillanpää *et al.* (2009)
Ace	*Enterococcus faecalis*	AF260889	43/143 (30)	70/143 (48)	Nallapareddy *et al.* (2000)

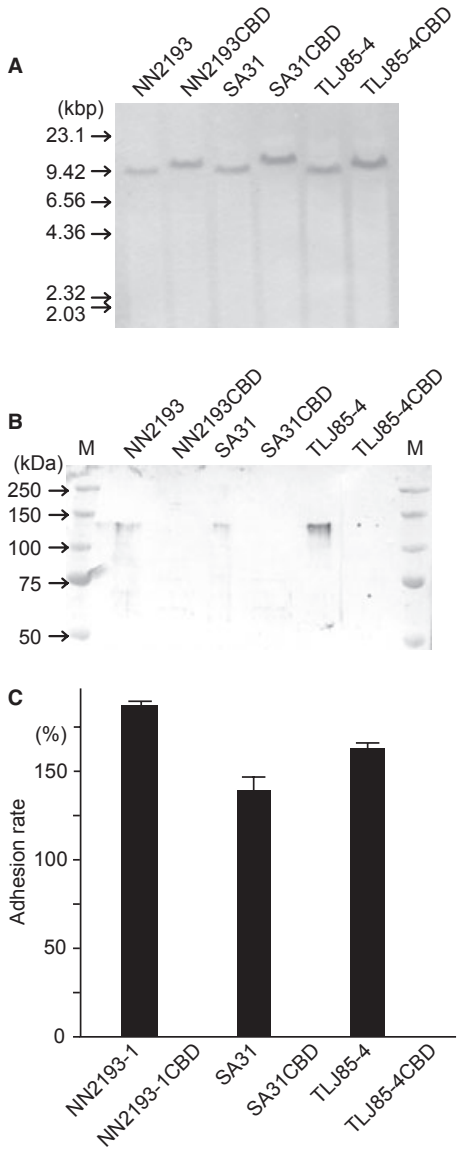

Figure 2 Construction of *cbm*-inactivated isogenic mutant strains. (A) Representative results of Southern hybridization of the *cbm* gene. (B) Representative results of Western blotting of the Cbm using antiserum against recombinant Cbm. (C) Collagen-binding properties of the strains standardized to that of TW871 which was set at 100%.

appropriate insertion of the erythromycin resistance gene (Fig. 2A) and was also confirmed by sequence determination. Western blotting analyses of Cbm in these strains showed no detectable expression of Cbm (Fig. 2B), and *in vitro* analyses indicated that these mutant strains lacked the ability to bind to type I collagen (Fig. 2C). In addition, the whole cells as well as those from urea extracts of Cbm-positive

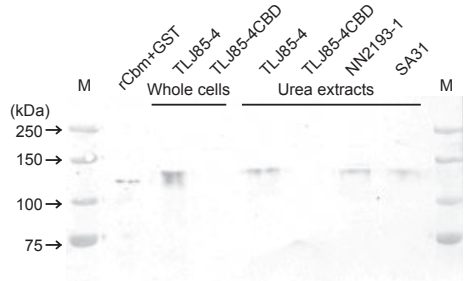

Figure 3 Western blotting of cell-surface proteins of Cbm-positive strains using Cbm-antiserum.

strains showed positive reaction for anti-Cbm in Western blot analyses (Fig. 3). The complemented mutant strain of SA31Comp recovered the binding activity of type I collagen (Fig. 4A). Also, the recombinant Cbm was demonstrated to inhibit collagen-binding of AT1 in a dose-dependent manner (Fig. 4B), and the inhibition was greater than with Cnm (Fig. 4C).

Distribution of the strains with collagen-binding properties

Table 2 shows a list of 19 serotype *k* strains analysed in the present study, among which two were blood isolates from patients with bacteremia and IE. The distribution of the strains with collagen-binding properties was shown to be approximately 85% of the total number, of which six and 10 strains were demonstrated to possess *cnm* and *cbm* genes, respectively. On the other hand, the remaining 15% of the serotype *k* strains showed negative collagen-binding *in vitro* and all of these strains lacked both *cnm* and *cbm* genes. We found five other strains with *cbm* genes, which were composed of one serotype *c*, three serotype *e* and one serotype *f* strains (Table 3). When comparing the collagen-binding properties of *cnm*-positive and *cbm*-positive strains, the collagen-binding activities of the *cbm*-positive group were significantly greater than those of the *cnm*-positive group ($P < 0.001$) (Fig. 5).

The *S. mutans* serotype distribution in Japanese children and adults were quite similar, with approximately 70–75% serotype *c*, followed by serotype *e* with approximately 20% (Table 5). As for Thai and Finnish subjects, the serotype distribution rates were also quite similar to Japanese subjects. Analysis of 580 *S. mutans* clinical isolates from 580 individuals revealed that the *cnm*-positive and *cbm*-positive

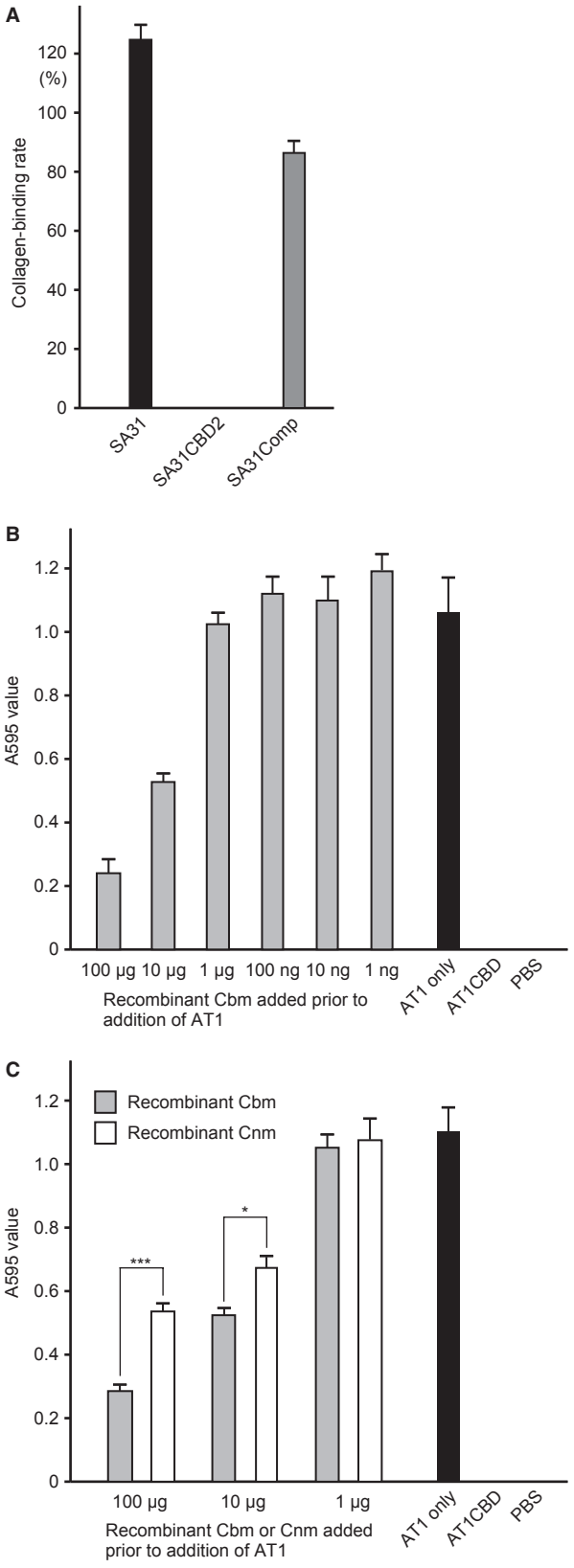

Figure 4 Molecular biological analyses of Cbm. (A) Collagen-binding activity of SA31, its *cbm*-inactivated mutant (SA31CBD2) and its complemented mutant (SA31Comp). (B) Recombinant Cbm (1 ng to 100 µg) was added to AT1 for binding evaluations by the same method used for the assay in Figure 2C. (C) Inhibition assays using recombinant Cbm or Cnm (1 µg to 100 µg) added to AT1 were also evaluated. There were statistically significant differences between the two groups (Student's *t*-test; $^{*}P < 0.05$ and $^{***}P < 0.001$).

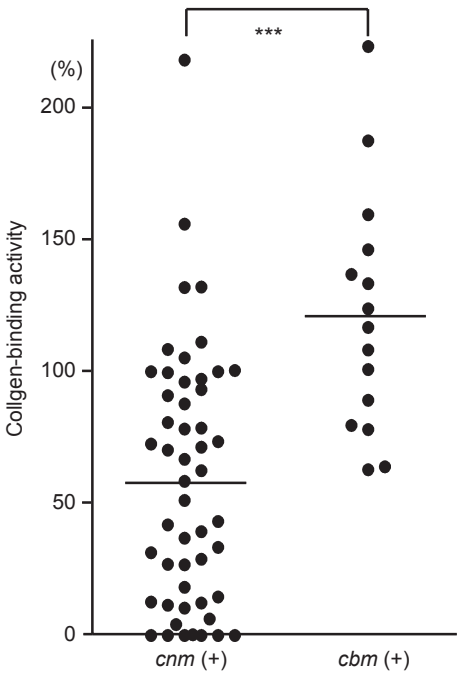

Figure 5 Comparison of the collagen-binding activities of *cnm*-positive ($n = 52$) and *cbm*-positive ($n = 15$) strains. The activities of these strains were expressed as the relative percentage standardized to that of TW871 at 100%. Horizontal lines indicate the mean values in each group. There were statistically significant differences between the two groups (Student's *t*-test; $^{***}P < 0.001$).

strains could be identified in approximately 10 and 2%, respectively (Table 6). In addition, the *cnm*-positive and *cbm*-positive strains were shown to be frequently identified in serotype *f* and serotype *k* groups, respectively ($P < 0.05$ and $P < 0.01$).

We then developed a multiplex PCR method to detect *cnm*-positive or *cbm*-positive strains (Fig. 6A). The estimated amplification sizes of the *cnm*- and *cbm*-positive specimens were 579 bp and 393 bp, respectively. The detection limit of this method was shown to be approximately 10–100 cells per reaction (Fig. 6B,C). When analysing 119 saliva specimens,

Table 5 Serotype distribution of clinical *Streptococcus mutans* oral isolates from 580 subjects

	Serotypes			
	c	*e*	*f*	*k*
Japanese subjects (*n* = 320)				
Children (*n* = 210)	160 (76.2%)	43 (20.5%)	4 (1.9%)	3 (1.4%)
Adult (*n* = 110)	80 (72.7%)	21 (19.1%)	5 (4.5%)	4 (3.6%)
Thai subjects (*n* = 150)	111 (74.0%)	29 (19.3%)	7 (4.7%)	3 (2.0%)
Finnish subjects (*n* = 110)	81 (73.6%)	20 (18.2%)	5 (4.5%)	4 (3.6%)
Total (*n* = 580)	432 (74.5%)	113 (19.5%)	21 (3.6%)	14 (2.4%)

Table 6 Distribution of the genes encoding collagen-binding adhesins in clinical oral isolates

	Serotypes				
	c (*n* = 432)	*e* (*n* = 113)	*f* (*n* = 21)	*k* (*n* = 14)	Total (*n* = 580)
cnm	37 (8.6%)	8 (7.1%)	15 (71.4%)[1]	4 (28.6%)	64 (11.0%)
cbm	1 (0.2%)	3 (2.7%)	1 (4.8%)	6 (42.9%)[2]	11 (1.9%)

[1]$P < 0.05$ and [2]$P < 0.01$ compared with all other serotypes (Fisher's exact probability test).

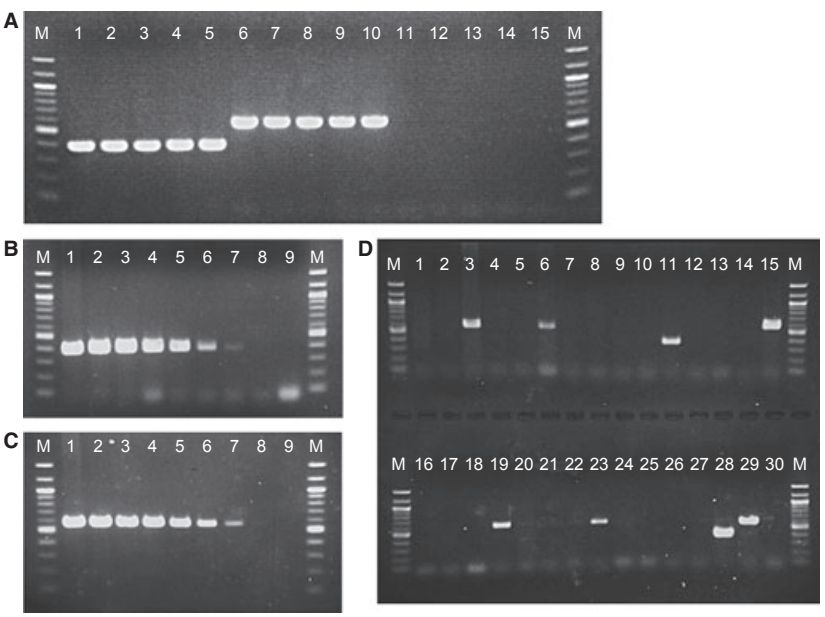

Figure 6 Construction of the multiplex polymerase chain reaction (PCR) method to identify subjects with *cbm*-positive and *cnm*-positive strains using saliva specimens. (A) The multiplex PCR method developed in the present study. The validity of the method was confirmed using several *cbm*-positive and *cnm*-positive strains. The positive bands for *cbm* and *cnm* are 393 bp and 579 bp, respectively. Lanes 1–5; *cbm*-positive strains, lanes 6–10; *cnm*-positive strains, and lanes 11–15; strains without *cbm* and *cnm*. M, 100-bp ladder. (B,C) The sensitivity of the PCR method for detection of *cbm* (B) and *cnm* (C). The sensitivity was examined by using titrated cultures with 10^8 cells per ml from strains TW295 and YT1. The following numbers of cells were added. Lanes: 1, 1×10^7; 2, 1×10^6; 3, 1×10^5; 4, 1×10^4; 5, 1×10^3; 6, 1×10^2; 7, 1×10^1; 8, 1; 9, sterile water. M, 100-bp ladder. (D) Representative results using saliva specimens. Samples in lanes 1 through 27 are specimens collected from 27 different individuals. Lanes: 28, YT1; 29, TW295; 30, sterile water. M, 100-bp ladder. The sample numbers 3, 6, 15, 19 and 23 were shown to be *cnm*-positive and sample number 11 was *cbm*-positive.

the frequencies for *cnm* and *cbm* were 16.3 and 1.3%, respectively (Fig. 6D).

Adhesion of *S. mutans* with collagen-binding properties to HUVEC

The adhesion for the three tested *cnm*-positive strains ranged from 7.7% to 19.8%, whereas their isogenic mutants showed drastic reductions in adhesion (Fig. 7). The adhesion rate of *cbm*-positive strains AT1, NN2193-1 and TLJ85-4 were approximately 25% whereas their Cbm-deficient mutants exhibited marked reductions in adhesion.

DISCUSSION

Infective endocarditis is one of the well-known diseases associated with dentistry, in which invasive dental procedures, such as extraction, could induce the onset of the disease at the impaired endothelium in the heart (Nakano & Ooshima, 2009). Recent surveys reported that staphylococci are more often identified in western countries, whereas oral streptococci are regarded as the major pathogens in Japan (Nakatani *et al.*, 2003; Moreillon & Que, 2004). Although the mitis group of streptococci is known to be the major causative agent for IE, there have been many IE cases induced by *S. mutans*, a major pathogen of dental caries (Lockwood *et al.*, 1974; McGhie *et al.*, 1977; Moore *et al.*, 1977; Robbins *et al.*, 1977; Smith *et al.*, 1977; Vose *et al.*, 1987; Ullman *et al.*, 1988; Gauduchon *et al.*, 2001; Nomura *et al.*, 2006). In our previous studies, the serotypes of four blood isolates of *S. mutans* were analysed and showed that none of the strains belonged to serotype *c*, the major serotypes in the human oral cavity (Fujiwara *et al.*, 2001). In subsequent studies, the sugar components of the serotype-specific polysaccharide of the two untypeable strains were specified and designated as a novel serotype *k* (Nakano *et al.*, 2004a). Recently, we performed a molecular analysis of extirpated heart valves from IE cases and found that bacterial DNA specific for serotype *k* was frequently identified (Nakano *et al.*, 2007c). Although the serotype *k* strains were shown to be resistant to phagocytosis by human polymorphonuclear leukocytes (Nakano *et al.*, 2004a), the molecular basis for the etiology of IE remains to be elucidated.

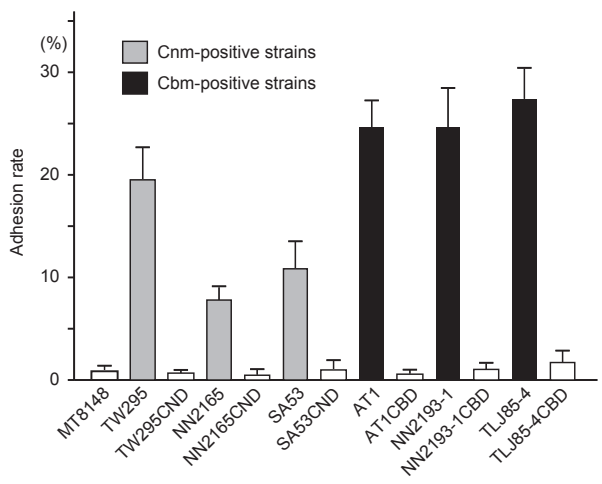

Figure 7 Adhesion properties of *Streptococcus mutans* strains to human umbilical vein endothelial cells. Adhesion was calculated based upon adherent bacteria relative to the total numbers of infecting bacterial cells. Data are expressed as the mean ± standard deviation of triplicate experiments. Closed and open columns are wild-type and its isogenic mutant strains, respectively.

It is known that IE initiates vegetative formations (composed of platelets, fibrin and bacteria) on the impaired endothelium. Hence, the abilities to induce platelet aggregation and to bind extracellular matrix proteins, such as collagen type I, laminin and fibronectin, are known to be important virulence properties of pathogenic bacteria involved in IE (Moreillon & Que, 2004; Nagata *et al.*, 2006). Therefore, we focused our attention on the type I collagen binding properties of the serotype *k* strains in the present study. Although the Cnm protein of *S. mutans* was previously demonstrated to be associated with the collagen-binding properties of *S. mutans*, we found that there are *S. mutans* strains with collagen-binding properties but with no *cnm* gene. This finding led us to consider the possibility that other protein antigens associated with collagen-binding properties could be present among these strains. We now report the identification of a distinct protein with high homology to Cnm and name it Cbm.

Cbm-defective mutant strains were demonstrated to lack collagen-binding activities and its complemented mutant recovered the binding properties. In addition, urea extracts of whole cells produced positive reactions with anti-Cbm in Western blot analysis. Hence, we concluded that Cbm, in addition to Cnm, is also a cell surface collagen-binding protein of *S. mutans*. It is of interest that Cbm displays a similar

structure to Cnm in that Cbm also possesses a collagen-binding domain and B-repeats. In addition, the entire length of the *cbm* gene was variable depending on the number of repeats, which is also similar to the *cnm* gene. Taken together, these results indicate that the molecular characteristics of protein Cbm are similar to Cnm. It is of interest to consider the relationship between Cbm and Cnm. It is possible to speculate that one of these genes may be the precursor of others and undergoes genetic alterations. However, other than this possible relationship it is currently difficult to speculate further.

The molecular weight of Cnm is reported to be approximately 120 kDa (Sato *et al.*, 2004), and that of Cbm is similar, which is reasonable in that the length of the open reading frames in Cbm and in Cnm are almost the same. In fact, Western blot analyses using anti-Cbm serum demonstrated that the positive bands in whole cells and urea extracts were found at the position of approximately 120 kDa. However, the positive bands of recombinant Cbm and its GST fusion were observed at apparently lower molecular size positions. This was unexpected and may be because one is native and the other is a recombinant protein. Additional approaches will be necessary to resolve this apparent paradox.

Streptococcus mutans has been reported to be detected in cardiovascular specimens by molecular biological analyses (Kozarov *et al.*, 2006; Nakano *et al.*, 2006; Nakano *et al.*, 2009), and has been studied in relation to cardiovascular diseases. The organisms also display the properties of adhesion to vein endothelial cells and invasion into arterial endothelial cells (Abranches *et al.*, 2009; Nakano *et al.*, 2010a; Nagata *et al.*, 2011). In the present study, the collagen-binding adhesins of *S. mutans*, such as Cnm and Cbm, were shown to be involved in adhesion to HUVEC. The strains with Cnm and Cbm were shown to be predominantly identified in serotype *f* and *k* strains, respectively, indicating that the strains of the minor *S. mutans* serotypes present in the oral cavity are highly virulent if they invade the bloodstream. This hypothesis is supported by the observations that serotypes *f* and *k* were identified with high frequency in *S. mutans*-positive cardiovascular specimens (Nakano *et al.*, 2007b,c).

The serotype distribution of *S. mutans* in the oral cavity and that in blood was demonstrated to be different, which might be a result of selection based upon the relative survival of specific type strains in blood (Nakano *et al.*, 2007b). In fact, molecular analyses of *S. mutans*-positive extirpated heart valves from IE patients showed a positive high-frequency correlation for serotype *k* strains (Nakano *et al.*, 2007c). Cell-surface structures, such as the approximately 190-kDa antigen (PAc) and several types of Gbp in serotype *k* strains were shown to be distinct from those of the strains commonly present in the oral cavity (Nakano *et al.*, 2008). The susceptibility to phagocytosis by polymorphonuclear leukocytes in serotype *k* strains was demonstrated to be low because of a defect in the glucose side-chain in the serotype-specific polysaccharide (Nakano *et al.*, 2004a). In addition, the PAc-defective mutant strains were also shown to be resistant to phagocytosis and the high-frequency isolation of strains with defects in this protein antigen was demonstrated (Nakano *et al.*, 2006). These observations suggested that serotype *k* strains could survive in the bloodstream for longer durations compared with other serotype strains. In addition, the adhesion properties to HUVEC by serotype *k* strains, such as TW295 and TW871, were shown to be approximately 15 times higher than the typical oral strains (Nakano *et al.*, 2010a). It is of interest that the present study demonstrated that the distribution frequency of the strains with Cbm is higher in serotype *k* strains. In addition, a defect in Cbm resulted in drastic reductions in adhesion to HUVEC. These results suggest that serotype *k* strains might be more virulent when they have the opportunity to contact exposed collagen tissue in blood.

The present study clearly demonstrated that the distributions of each serotype in three countries were similar. In addition, strains positive for *cbm*, as well as *cnm*, were identified in the isolates from all countries examined. Hence, we speculate that the *cbm*-positive and *cnm*-positive strains are prevalent worldwide. In the present study, we developed a simple multiplex PCR method to identify these strains using saliva specimens. We plan to investigate the distribution of these strains worldwide in subsequent studies. It might be possible that identification of these strains might specify subjects at risk for IE caused by *S. mutans* when they have underlying heart disorders associated with IE risk. Further research should focus on the analyses of clinical specimens collected during surgical treatment of IE.

In summary, a collagen-binding adhesin, Cbm, was characterized and its coding gene *cbm* was also sequenced in the present study. The *S. mutans* strains with Cbm are predominantly identified in serotype *k* strains, which are minor serotypes in the oral isolates. Furthermore, *S. mutans* strains with Cbm may be prevalent worldwide. The collagen-binding properties of the strains with Cbm are significantly higher than those strains with Cnm proteins. In addition, these collagen-binding proteins are associated with adhesion to HUVEC. These results suggest that Cbm, as well as Cnm, may be associated with the relative enhanced virulence of *S. mutans* in blood-borne diseases.

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