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Identification of a glucan-binding protein C gene homologue in *Streptococcus macacae*

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Background: The past few decades have seen the isolation of certain glucosyltransferases and a number of proteins from mutans streptococci. Some of these proteins have been shown to possess glucan-binding capabilities which confer an important virulence property on mutans streptococci for the role of these bacteria play in dental caries. Among these proteins is glucan-binding protein C, which is encoded by the *gbpC* gene, and which we have identified as being involved in the dextran-dependent aggregation of *Streptococcus mutans*. However, *gbpC* homologues have yet to be identified in other mutans streptococci.
Methods: We carried out polymerase chain reaction amplification of *Streptococcus macacae* using primers that were designed based on conserved sequences of *S. mutans gbpC* and identified a *gbpC* gene homologue. The gene of that homologue was then characterized.
Results: Nucleotide sequencing of the *S. macacae gbpC* homologue revealed a 1854 bp open reading frame encoding a protein with an N-terminal signal peptide. The molecular mass of the processed protein was calculated to be 67 kDa. We also found an LPxTG motif, the consensus sequence for gram-positive cocci cell wall-anchored surface proteins, which was followed by a characteristic sequence at the carboxal terminal region of the putative protein. This suggests that the *S. macacae* GbpC homologue protein was tethered to the cell wall.
Conclusion: Based on these results, together with the demonstrated glucan-binding ability of the *S. macacae* GbpC homologue protein, we suggest that *S. macacae* cells are capable of binding dextran via the GbpC homologue protein, which is similar to the *S. mutans* GbpC protein. In addition, Southern hybridization analysis using the *S. macacae gbpC* homologue as a probe showed a distribution of *gbpC* homologues throughout the mutans streptococci.

Key words: *Streptococcus macacae*; *gbpC* gene homologue; glucan-binding; aggregation; mutans streptococci

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Mutans streptococci have been implicated as the most important microbial agents in dental caries (14). One important virulence property of these organisms is their ability to adhere to tooth surfaces by producing several extracellular proteins or enzymes (14). In a previous study, we identified and characterized *Streptococcus mutans* glucan-binding protein C (GbpC) as a wall-anchored surface protein characteristic of gram-positive bacteria (29). This protein probably participates in the adherence to tooth surfaces where mediated by α-1,6 glucan (dextran), and therefore may contribute to the cariogenicity of these organisms (16, 18, 27). Four genes encoding glucan-binding proteins in *S. mutans* have been identified so far and designated in the order of their discovery as *gbpA* (3), *gbpB* (17), *gbpC* (29), and *gbpD* (32). Among these glucan-binding proteins, only the GbpC protein is involved in dextran-dependent aggregation (ddag), defined as the ability to immediately autoaggregate in the presence of exogenously added dextran (15). *Streptococcus sobrinus* has been found to aggregate more readily than *S. mutans* (7, 38), and several glucan-binding proteins have been reported in *S. sobrinus*, including candidates involved

in ddag (15, 34). However, no glucan-binding protein genes have been identified in *S. sobrinus* or any other mutans streptococcus, except for *S. mutans*.

Streptococcus macacae, isolated from the macaque monkey, is a species of mutans streptococci (5). Its cariogenicity, however, has yet to be experimentally verified. As a mutans streptococcus, *S. macacae* exhibits several relevant cariogenic properties, including sucrose-dependent smooth surface attachment, acidogenicity, and colonial morphology specific to mutans streptococci on Mitis-Salivarius agar plates. This organism is more similar to *S. mutans* than other mutans streptococci with respect to serotype polysaccharide antigens and genomic GC content (5). However, only a few nucleotide sequences, e.g. those encoding 16S ribosomal RNA, elongation factor Tu (*tuf*), Cnp60 (*cnp60*), and ribonuclease P have been determined in *S. macacae* so far (accession numbers AF276268, AY123711, AJ511702, and X58302, respectively). There are no reports characterizing the genes responsible for the cariogenic properties of *S. macacae*. In the present study, we found that *S. macacae* also exhibited ddag, and attempted to detect a *gbpC* homologue in the *S. macacae* chromosome by polymerase chain reaction (PCR) using primers designed based on the *gbpC* nucleotide sequences determined from 19 strains of *S. mutans* (26). To investigate the universality and distribution of *gbpC* homologous genes in mutans streptococci, we performed Southern hybridization analysis on seven species of mutans streptococcus using an *S. macacae gbpC* homologue fragment probe.

Material and methods

Bacterial strains and plasmids

S. macacae strain ATCC 35911 (serotype c) was used for the identification and cloning of the *gbpC* gene homologue and glucan-binding assays. *S. mutans* strain 109cS (serotype c), a spontaneous colonization-defective mutant of 109c formed by homologous recombination between the *gtfB* and *gtfC* genes (29), and *S. sobrinus* strain ATCC6715 were used as references for ddag. *S. mutans* strain Z1, which is a ddag-defective mutant from a single nucleotide deletion resulting in a frameshift mutation in *gbpC* (accession number AB195694), was used for the complementation test. In addition, the following strains were subjected to Southern hybridization analysis: *Streptococcus criceti* strains HS6 (ATCC19642) and OMZ61, *Streptococcus ratti* strains 107P and FA-1 (ATCC19645), *S. mutans* strain UA159 (ATCC700610), *S. sobrinus* strain 100–4 (clinical isolate), *Streptococcus downei* strain MFe28 (ATCC33748), *Streptococcus ferus* strain 8S1 (ATCC33477), *Streptococcus sanguinis* strain ATCC10556, *Streptococcus gordonii* strain Challis, *Streptococcus anginosus* strain IS57, and *Streptococcus salivarius* strain ATCC9222. The streptococci were maintained and cultured in Todd–Hewitt broth/agar plates. *Escherichia coli* strain TOP10 obtained from a commercial supplier (Invitrogen, Carlsbad, CA), was used as a host for plasmid pBAD/His and its derivatives.

Dextran-dependent aggregation

S. macacae was streaked onto Todd–Hewitt agar plates and aerobically incubated at 37°C for 2 days. Colonies were picked and cultured in Todd–Hewitt broth overnight at 37°C without shaking. The culture was then transferred as a 1% inoculum into screw-capped glass tubes containing fresh BTR-G broth (1% Bacto Tryptone, 0.1% Bacto yeast extract, 0.05% sodium thioglycolate, 0.61% K_2HPO_4, 0.2% KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $MnSO_4$, 0.2% glucose) (31). As reference strains, *S. mutans* 109cS and *S. sobrinus* ATCC6715 were grown in BTR-G broth with and without a sublethal concentration (0.18 μg/ml) of tetracycline (tet) as a stress inducer, respectively. The cultures were incubated for 24 h and then divided into two 1.0-ml portions and a solution of dextran T2000 (molecular weight, 2000,000; Sigma-Aldrich Co., St. Louis, MO) added to one of them to a final concentration of 100 μg/ml. Each pair of tubes was swirled for more than 2 min and observed visually for aggregation (29).

Southern hybridization analysis

The streptococcal strains were subjected to agarose gel electrophoresis and the appropriate restriction enzyme-digested chromosomal DNA fragments were separated and transferred onto nylon membranes (NytranN, Schleicher and Schuell, Dassel, Germany) as described previously (23, 30). The chromosomal fragments were hybridized with an *S. mutans* intact *gbpC* gene or *S. macacae gbpC* homologue fragments as probes and then analyzed using the Enhanced Chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System recommended by the supplier (Amersham Biosciences Corp., Piscataway, NJ).

The *S. mutans gbpC* 1944 bp gene fragment containing the intact gene to be used as a probe was amplified by PCR from *S. mutans* 109cS chromosomal DNA with forward primer gbpC5′ (5′-GAGA-AAGCACTTTGGTTTCAA-3′) and reverse primer gbpC3′ (5′-ACATATTT-TTCTCCAACCCTG-3′). The *S. macacae gbpC* homologue fragment probe was amplified from *S. macacae* ATCC 35911 chromosomal DNA with forward primer SMA-FuF (5′-ATCTCGAGGCTGCTC AGCAAATGTT-3′) and reverse primer SMA-FuR (5′-ATGAATTCAGTTTG-AGG AAGCGGAGCA GTATA-3′).

PCR amplification of *gbpC* homologue from *S. macacae* chromosome

The primers were designed based on the codon bias of *S. macacae* and the conserved sequences which were revealed by comparing 19 *gbpC* sequences from different strains of *S. mutans* (DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers D85031, NC_004350, and AB195694-AB195710). The PCR primers used in this study are listed in Table 1. Each PCR mixture (50 μl) consisted of 1× cloned *Pfu* reaction buffer, 200 μM dNTPs, 0.2 μM oligonucleotide primers, 2.5 U of *PfuTurbo* hot start DNA polymerase (Stratagene Corp., La Jolla, CA), and 5 μl template (100 ng/ml *S. macacae* chromosomal DNA). The PCR thermal conditions were as follows: activation at 95°C for 2 min, 30 cycles of 3-step amplification (95°C for 30 s, 54°C for 30 s, and 72°C for 70 s), followed by a final extension at 72°C for 10 min. Agarose gel (1.5%) electrophoresis was used to analyze the PCR products.

Chromosome walking

A genome walking library was constructed with the Universal GenomeWalker Kit (Clontech, Palo Alto, CA) and the *Bam* HI, *Pvu*II, and *Ssp*I restriction sites confirmed by Southern hybridization analysis. The initial amplicon was used for the Southern hybridization analysis as a probe against *S. macacae* chromosomal DNA. The *S. macacae* chromosomal fragments flanking the initial amplicon were then amplified with an adopter primer (AP1) and gene specific primers, as recommended by the supplier. The amplified fragments were then purified and sequenced using the same primers. Based on the obtained nucleotide sequence information, subsequent forward and reverse primers were designed and appropriate fragments

Table 1. Primers used in identification and sequencing of *Streptococcus macacae gbpC* gene homologue and in complementation test. SMA-YF1, SMA-YF2, SMA-YR1, SMA-YR2, and SMA-YR3 were used to detect *S. macacae gbpC* homologues. AP1, SMA-KF4, SMA-KR5, SMA-KR3, SMA-KF5, SMA-KR1, SMA-KF1, SMA-KR7, SMA-KF2, SMA-KF9 and SMA-KR9 were used in genome-walking. Numbers following names correspond to position in Fig. 1. Primer AP1 was supplied with Universal Genome Walker Kit (Clontech). Primers cnmChiF, cnmChiR, cnmCRT, SmaChiF, SmaChiR, SmaCFT, SmaCRT, DSHpa3F, cnmchkR, P8 and P8CFT were used in complementation test. Primer P8 was supplied by TOYOBO. Numbers following sequences correspond to position in *S. mutans* strain Z1 *cnm* gene sequence under accession number AB102689. Location of cnmchkR primer is 223 bp downstream of AB102689 sequence.

Name	Sequence (5′- -3′)	Primer orientation
AP1	GTAATACGACTCACTATAGGGC	
SMA-YF1	GAAGATACTTGGTT**Y**AA**R**ATGAA	Forward
SMA-YF2	ATGAGTCTCTCATCGTTAAA**Y**CA**Y**TGGAC	Forward
SMA-YR1	TCATTGCCATTTTCATC**R**TA**R**AA	Reverse
SMA-YR2	GTCATAGCACCTGCACC**R**TA**R**TA	Reverse
SMA-YR3	TCAACAACAAGATT**Y**TT**R**TGCCA	Reverse
SMA-KF4 (1)	GCTAATACTGACAAGAGTGGAC	Forward
SMA-KR5 (2)	AGGAGAATCTGTCTCTTGCTTA	Reverse
SMA-KR3 (3)	TGAGCTGTTTCCACTCGGC	Reverse
SMA-KF5 (4)	AGCTCAGTCACTGGTTTTT	Forward
SMA-KR1 (5)	GTTCAACAAGAGTCGTACCATCATTGC	Reverse
SMA-KF1 (6)	GCCAATGGTGCAGCTTTTAAT	Forward
SMA-KR7 (7)	GCTCTCTCACTCGCACCTCA	Reverse
SMA-KF2 (8)	CGAGTGAGAGAGCGCCA	Forward
SMA-KF9 (9)	GATGTAAAAGAAGTTCGAGA	Forward
SMA-KR9 (10)	CCTGACGTTTTTTAAGTTCTTC	Reverse
cnmChiF	ATGAAAAGAAAAGGTTTACGAAGACTATTA (68-97)	Forward
cnmChiR	ATCATTAATCTGAGTCGGTGATACC (220-196)	Reverse
cnmCRT	pGCCGCCATCATTAATCTGAGTCGGTGATACC	Reverse
SmaChiF	GCTGCTGCTCAGCAAAATG	Forward
SmaChiR	TTAGTTCTCTTTTTTACGTCTTTTAGCT	Reverse
SmaCFT	pGGCGGCGCTGCTGCTCAGCAAAATG	Forward
SmaCRT	pGACGACTTAGTTCTCTTTTTTACGTCTTTTAGCT	Reverse
DSHpa3F	AAACAACTACTAAAGTTAAGGAACCAGA (1473-1500)	Forward
cnmchkR	AATCATTTTTTCTTCATTATAAAAAGC	Reverse
P8 (TOYOBO)	AGCGGATAACAATTTCACACAGGAAAC	Forward
P8CFT	pGTCGTCAGCGGATAACAATTTCACACAGGAAAC	Forward

Abbreviations: **R** (A/G),**Y**(C/T),**N** (*A*/T/G/C), **Glu**: GA**R**, **Asp**: GA**Y/R**TA, **His/Gln**: CA**N**, **Phe**: TT**Y/R**AA, **Lys**: AA**R/Y**TT, **Asn**: AA**Y**, **His**: CA**Y/R**TG, **Tyr**: TA**Y/R**TA.

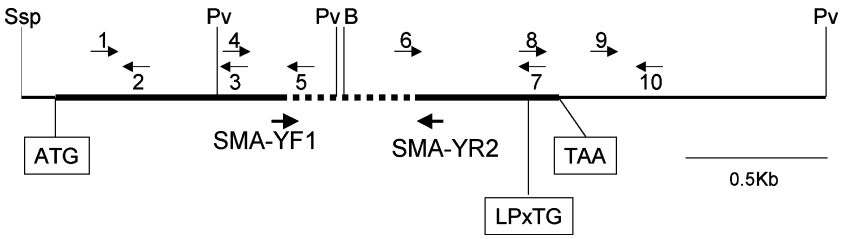

Fig. 1. Restriction map around *gbpC* homologue and locations of primers designed for identification of ORF. Thick-line indicates region of *gbpC* gene homologue. Dotted line indicates region which was obtained by initial PCR amplification and subsequent sequencing with primers SMA-YF1 and SMA-YR2. Arrows with numbers indicate approximate PCR primer locations and their directions (5′ → 3′). Other symbols are: B, *Bam*HI; Pv, *Pvu*II; Ssp, *Ssp*I indicating respective restriction enzyme recognition sites.

were amplified for sequencing. The locations and sequences of the primers used are indicated in Fig. 1.

Nucleotide sequencing and sequence analysis

The amplified fragments were purified with a QIAquick PCR Purification Kit (Qiagen K.K., Tokyo, Japan). The corresponding regions were directly sequenced with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit using the primers listed in Table 1 and an ABI PRISM Genetic Analyzer 310 (Applied Biosystems, Foster City, CA) as described previously (27). The nucleotide sequences of both strands of the 2.6 kb fragments encompassing the *S. macacae gbpC* gene homologue were then determined. 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 analyses and multiple alignments were carried out with DNASIS-Mac (Hitachi Software Engineering, Yokohama, Japan) and GENETYX-MAC (Genetyx Corporation, Tokyo, Japan) programs.

Cloning and expression of *S. macacae* GbpC homologue protein

A gene fragment expressing the *S. macacae* GbpC homologue but not the region encoding the signal sequence predicted by the SIGNALP program 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (20) was amplified by PCR using forward primer SMA-FuF2 (5′-ATCTCGAGGTTTTTGCAGATGATATCAATGCTGC-3′) and reverse primer SMA-FuR (5′-ATGAATTCAGTTTGAGGAAGCGGAGCAGTATA-3′). It was then ligated inframe to the 5′ histidine-tag region (the *Xho*I and *Eco*RI sites utilized are underlined in the sequences) of expression vector pBAD/HisA (Invitrogen). Following transformation with *E. coli* strain TOP10, the resulting clones were analyzed as described previously (23). One of these clones, pBAD-SmaGbpC, was used to perform glucan-binding assays along with strain TOP10 harboring vector pBAD/HisA (strain pBAD) as a control. The cells of these strains grown with or without 2×10^{-3}% arabinose as an inducer were collected, washed, and subjected to 6 cycles of ultrasonication to obtain crude cell-free extracts for the glucan-binding assays as described previously (28). Before the assays, induction of histidine-tagged proteins was confirmed with SDS-PAGE and Coomassie Brilliant Blue R (CBBR) staining. The proteins were also analyzed by Western blot analysis using anti-GbpC antibody. The five gene fragments expressing the partial GbpC homologue proteins (pBAD-FraA, B, C, D, and E) were also constructed as described above. pBAD-FraA and pBAD-FraB are the homologous regions of *S. mutans* GbpC fragment A and fragment B, respectively (Matsumoto, personal communication). pBAD-FraC, pBAD-FraD, and pBAD-FraE were designed based on the predicted secondary structure of the

S. macacae homologue protein. The primers used for amplifying the fragment A homologous region were forward primer SMA-FraAFW (5′-ATCTCGAGGAAGCTGCAGATGCTGATAATAA-3′) and reverse primer SMA-FraARV (5′-ATGAATTCTTATGAGTAGTTCACGGATTTAGGAG-3′). The primers used for amplifying the fragment B homologous region were forward primer SMA-FraBFW(5′-ATCTCGAGGCTGGCCGAGTGGAAA-3′) and reverse primer SMA-FraBRV (5′-ATGAATTCTTAGCCTAAATTGACCTTCTCTATATGG-3′). The forward primers used for amplifying the fragment C, D, and E regions were SMA-FraCFW (5′-ATCTCGAGCAAAAAGAAGTAGCAGCTGGTAAA-3′), SMA-FraDFW (5′-ATGAATTCTTAATGACTATTGAAGGTGTCAGCCAC-3′), and SMA-FraEFW (5′-ATCTCGAGAATTATTCTAATTCAGACTATGTCAGTCAA-3′), respectively. Reverse primer SMA-FraARV was also used to amplify fragment C, D, and E regions. The appropriate concentrations of arabinose as an inducer were 2×10^{-2}% for fragments A, C, D, and E, and 2×10^{-3}% for fragment B.

Glucan-binding assay

Glucan-binding assays were carried out using biotin-dextran (dextran, biotin, 70,000 molecular weight, lysine fixable; Molecular Probes Europe BV, Leiden, the Netherlands) as described previously (13), but modified to utilize the Ni-affinity of the histidine tag to immobilize the proteins using the method of Shah & Russell (32).

The crude extracts from the induced *E. coli* strain cells with the overexpression plasmids or vector controls were prepared as described above. Preliminary experiments revealed that > 10 μl of crude extract was sufficient to saturate the binding capacity of Ni-NTA-coated wells (data not shown). Therefore, 50 μl of crude extract was added to the Ni-NTA-coated 96-well HisSorb Plates (Qiagen K.K.) in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBST) to a final volume of 200 μl and incubated overnight at 4°C. The protein solutions were then removed and the wells were given four 1-min washes with 200 μl PBST. A 200 μl solution of biotin-dextran in PBS + 0.2% bovine serum albumin (PBSB) was added and incubated for 10 min. After washing as described above, the wells were incubated with 200 μl of a 1 : 500 dilution of Streptavidin-horseradish peroxdase conjugate (Amersham Biosciences Corp.) in PBSB for 30 min and then given four 1-min washes with 200 μl PBST. An aliquot of 100 μl *o*-phenylenediamine dihydrochloride was used as a substrate for color development of the solutions. After incubation with the substrate solution for 1.5 min, 50 μl of 2 N H_2SO_4 was added to stop the reaction, and the resulting A_{490} was then measured in a microplate reader (Bio-Rad MICROPlate READER model 3550, Bio-Rad, Hercules, CA).

For competition studies, 100 μl of competitors (unlabeled dextran: Dextran D-1537, 72,400 molecular weight, and amylose: Amylose A-0512, both Sigma-Aldrich Co.) were added to 100 μl of biotin-dextran solution in PBSB and the plates incubated at room temperature for 10 min, followed by treatment as described above.

Next, the affinity of α-1,3 glucan (kindly provided by Professor Kodama, Kyushu Institute of Technology) for the *S. macacae* GbpC homologue protein was examined. PBST 400 μl containing a volume corresponding to 0.1 μl crude extract was prepared and divided into two 200-μl portions. α-1,3 glucan 20 mg were added to one tube after swirling for 5 min. Glucan-binding assays were then performed as described above, and the affinity of the α-1,3 glucan for the *S. macacae* GbpC homologue protein was monitored for glucan-binding activity and compared with the α-1,3 glucan added to the protein-deficient sample.

SDS–PAGE and Western blot analysis

The crude extracts from the induced *E. coli* cells with the overexpression plasmids or vector controls described above were mixed with SDS sample buffer and frozen until electrophoretic analysis using the Laemmli-gel system with 5–20% acrylamide gels (SuperSep™, Wako, Osaka, Japan). Following SDS-PAGE, the separated proteins were transferred to PVDF membrane (Immobilon-P™, Millipore Corporation, Bedford, MA). Western blot analysis was then carried out with either anti-Xpress antibody or antiserum generated against the *S. mutans* GbpC protein as described previously (29).

Complementation test in *S. mutans* strain Z1 by construction of chimeras *in vitro*

Constructing mutants of the *gbpC* homologue in *S. macacae* is difficult because of its nontransformable nature (unpublished results). Therefore, we constructed chimeric gene fragments composed of the *S. macacae gbpC* homologue and *S. mutans cnm* (25) by employing asymmetric PCR techniques which utilized 5′ phosphorylated primers *in vitro.* All PCR amplifications were carried out with high fidelity DNA polymerases – either *PfuTurbo* hot start DNA polymerase (Stratagene) or KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). Each of the PCR amplification products in this procedure was purified using a QIAquick PCR Purification Kit (Qiagen). The sequences of the primers used in this experiment are listed in Table 1.

The 180 bp *S. mutans cnm* 5′-gene fragment, including the region encoding the signal sequence, and the 2 kb fragment of the *S. macacae* intact *gbpC* homologue region were amplified using the PCR primers cnmChiF, and cnmChiR for the former, and SmaChiF and SmaChiR for the latter. These fragments were then used as templates following asymmetric PCR. The primers were designed for inframe ligation of both fragments. Asymmetric PCR using the primers cnmCRT and SmaCFT was performed using the above-mentioned PCR products as templates. PCR thermal conditions were 94°C for 12 s, 60°C for 30 s, and 67°C for 7 min using rTth DNA polymerase (Toyobo) to add 3′-adenine residue overhangs at the end of PCR products in order to diminish unwanted ligations. Primers cnmCRT and SmaCFT were 5′ phosphorylated and were designed to produce complementary single strands which protrude from asymmetric PCR products, allowing them to be annealed and ligated with each other. Ligation was performed overnight at 16°C with T4DNA ligase. The ligated fragments were subjected to PCR amplification with the primers cnmChiF and SmaChiR. The resulting 2.2 kb fragment (*S. mutans cnm*5′:: *S. macacae gbpC* homologue) was obtained and chimeric ORF was confirmed by nucleotide sequencing. The Em^r-*cnm*3′ fragment was prepared by ligating the 1.1 kb Em^r fragment upstream of the *cnm* 3′ fragment subcloned by TA cloning and subjected to PCR with P8 primer (Toyobo) and cnmChkR. In the same way as described above, a 3.8 kb fragment composed of the *S. mutans cnm*5′:: *S. macacae gbpC* homologue and the Em^r-cnm3′ was also constructed. This fragment was then transformed into ddag-defective *S. mutans* strain Z1, which we have previously found to exhibit cold agglutination (25). The colonies were screened following ddag and cold agglutination assays.

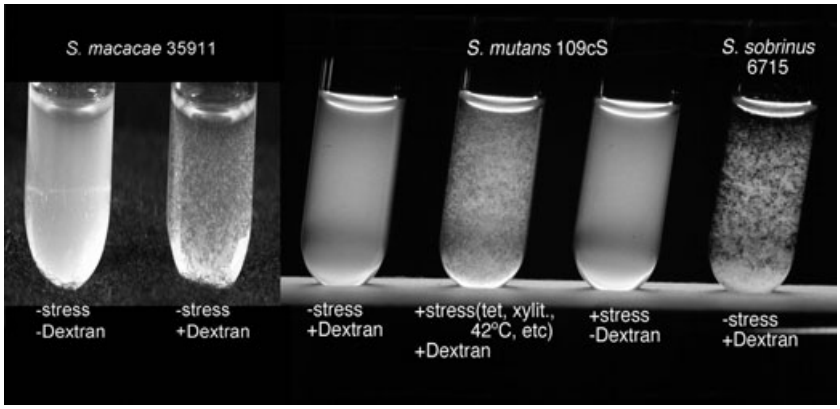

Fig. 2. Dextran-dependent aggregation of *S. macacae.* Dextran-dependent aggregation of *S. macacae* ATCC 35911, *S. sobrinus* 6715, and *S. mutans* 109cS grown in BTR-G broth with (+) or without (–) stress induced by 0.18 μg/ml tet or 0.6% Xylitol or grown at 42°C. Appearance of dextran-dependent aggregated cells of *S. sobrinus* 6715 and *S. mutans* 109cS also shown for reference.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB195308.

Results

Presence of homologue of *S. mutans gbpC* gene on *S. macacae* chromosome

Dextran-dependent aggregation in *S. macacae* was investigated as described in Material and methods. *S. macacae* exhibited ddag, as did *S. mutans* and *S. sobrinus,* which were used as reference strains (Fig. 2) (29). Although *S. macacae* did not autoaggregate as strongly as *S. sobrinus*, its aggregation properties were similar to those of *S. mutans*. The aggregation of *S. mutans* was dependent on the growth media used, and was difficult to detect with either Brain Heart infusion or Todd–Hewitt broth. In contrast, *S. macacae* cells exhibited aggregation upon addition of dextran even when grown in Todd–Hewitt broth under non-stress conditions. In this respect, this behavior was similar to that of *S. sobrinus*.

Southern hybridization analyses of *Pst*I- and *Hin*dIII-digested chromosomal DNA fragments from *S. macacae* chromosomal DNA revealed cross-hybridizing bands when probed with labeled *S. mutans* whole *gbpC* gene fragment, although the band intensities in the *S. macacae* were not as strong as those displayed by *S. mutans* (data not shown). Taken together with the results for ddag in *S. macacae*, this suggests that *S. macacae* possesses a homologue of the *S. mutans gbpC* gene.

Detection of *S. macacae gbpC* gene homologue by PCR amplification

The high stringency conditions of the Southern hybridization analysis of *S. macacae* chromosomal DNA using the *S. mutans gbpC* gene fragment as a probe yielded positive bands. For example, *Pst*I-digested *S. macacae* chromosome exhibited approximately 4 kb positive bands (data not shown). Therefore, we attempted to amplify an *S. macacae gbpC* gene homologue. We have previously detected a *gbpC* nonsense mutation in strain GS-5 (27). In addition to this mutation, several synonymous and nonsynonymous nucleotide substitutions have been detected in the gene of this strain. Polymorphism in the *gbpC* gene was expected, so we determined the 19 *gbpC* sequences from different strains of *S. mutans*. The results indicated many single nucleotide substitutions present throughout the gene. However, several relatively conserved stretches were revealed by multiple alignments of these sequences (26). Therefore, several PCR primers were designed based on the sequences in the conserved regions. The codon bias of *S. macacae* was also considered in designing the primers, along with the frequency of the third base position in the codon table. PCR amplification was carried out using several primer sets, as listed in Table 1. We successfully obtained two amplicons with overlapping sequences. The sequence of the longer amplicon was determined and revealed an approximately 70% homology to that of the corresponding region in *S. mutans* strain 109cS. The nucleotide sequence of the 2.6 kb fragment containing the entire *gbpC* homologous region was then determined.

Nucleotide and deduced amino acid sequences of *gbpC* homologue gene.

The 1854 bp open reading frame (ORF) begins with an ATG and terminates with a TAA codon. This ORF encodes a 617-amino acid protein with a calculated molecular mass of 67 kDa. The *gbpC* homologue gene exhibited a 67% identity with the *gbpC* in *S. mutans* strain 109cS (Fig. 3). The GbpC homologue protein has a 64% overall identity and 77% similarity with the *S. mutans* strain 109cS GbpC protein. Three putative candidates for a Pribnow box with relatively weak matches, e.g. TTGCAA-N17-TAA-TAT, were detected within 100 bp upstream of the ORF using a Promoter Prediction program by Neural Network (http://www.fruitfly.org/seq_tools/promoter.html) (17). A potential ribosome binding site (AAAAGGT) was found 9 bp upstream of the ATG initiation codon of the *gbpC* homologue gene and an inverted repeat was detected 30 bp downstream of the TAA termination codon with − 65.94 kJ of free energy per mol (35). This suggests that the *gbpC* gene homologue in *S. macacae* is monocistronic, similar to the *S. mutans gbpC* gene. The *gbpC* homologue gene product contained several features generally found in the surface proteins of gram-positive bacteria. The GbpC homologue amino-terminus codes for a signal peptide-like sequence with relatively high proportions of basic amino acids. This putative signal peptide would consist of 27 amino acids (20). The sequence LPQTG, which matches the consensus sequence LPxTG for the gram-positive cocci wall-anchored surface protein family, was detected at residue 581 of the protein, and the carboxal-terminal sequence, -KRRKKEN, was very similar to the membrane anchor region of various wall-anchored surface proteins found in gram-positive cocci (19). A possible cell wall-associated region was also found between amino acid residues 519 and 557, which contained 12 regularly distributed proline residues.

Another feature of the overall amino acid composition of this putative mature GbpC homologue protein was its relatively high alanine (11.18%) and proline (5.8%) content, which was similar to that of the 58–60 kDa glucan-binding lectin isolated from *S. sobrinus* by Ma *et al*. (15) (12.1% and 4.9%, respectively).

```
SmaGbpC     1 MKHKVGKVTLLSGLALAAAVSTTTVFADDINAAAQQNVDANTAAANTDKSGQASADDTQN   60
SmuGbpC     1 MKSKTAKITLLSSLALAA-FGATNVFADEAST----QLNSDTVAAPT-------------   42
                ** *  * **** *****    * ****            * ** *

SmaGbpC    61 SKAVNTDTAAAKTDNKQETDSPIVTVTDTKTEGQTTTTTSQVESKALNEAKAAAKEAKVK  120
SmuGbpC    43 -----ADTQASEPAATEKEQSPVVTVVESHTQGNTTTTTSQVTSKELEDAKANANQEGLE   97
                    ** *         ** ***    * * ******** ** *  *** *

SmaGbpC   121 ITETANQQQPSIEAADADNKVQAQAINKTVSDYNKAKEEYAQKQKQYQKDLEDYKSKKAE  180
SmuGbpC    98 VTETETQKQPSVEAADADNKAQAQTINTAVADYQKAKAEFPQKQEQYNKDFEKYQSDVKE  157
                ***  * *** ******** *** **  * ** *** *  *** ** ** * * *   *

SmaGbpC   181 YEIAKKAYEDYQKEVAAGKAAGRVETAQSLVFRSEPEATMTIEGVSQYLTKEARAANKPK  240
SmuGbpC   158 YEAQKAAYEQYKKEVAQGLASGRVEKAQGLVFINEPEAKLSIEGVNQYLTKEARQKHATE  217
                **  * *** * **** * * **** ** ***  ****   **** ********

SmaGbpC   241 DILEQFNTDNYSNSDYVSQNPYTPKEDTWFKMKVGDQITVTYNNIKNSSYMNKKISKVVS  300
SmuGbpC   218 DILQQYNTDNYTAADFTQVNPYDPKEDTWFKMKVGDQISVTYDNIVNSKYNDKKISKVKI  277
                *** * *****   *    *** *************** *** ** ** *  ******

SmaGbpC   301 TYKLNSTTSNDGTTLVELFHDPTKTIFIGAQTANEGRKDQISVTMQLTFFDENGDPINLS  360
SmuGbpC   278 NYTLNSSTNNEGSALVNLFHDPTKTIFIGAQTSNAGRNDKISVTMQIIFYDENGNEIDLS  337
                * *** * * *  ** *************** * ** * ******  * ****  * **

SmaGbpC   361 GNNAIMSLSSLNHWKTAYGDHIEKVNLGGNEFVKIPGSSVDLHGDTIYSANDNQYKANGA  420
SmuGbpC   338 SNNAIMSLSSLNHWTTKYGDHVEKVNLGDNEFVKIPGSSVDLHGNEIYSAKDNQYKANGA  397
                ************** * **** ****** ***************  **** *********

SmaGbpC   421 AFNGDGEDGWDAINEDGFPRAATAYYGAGAMTYKGQPFTFSVGGNDKGVPTTFWFSTNSV  480
SmuGbpC   398 TFNGDGADGWDAVNADGTPRAATAYYGAGAMTYKGEPFTFTVGGNDQNLPTTIWFATNSA  457
                ***** ***** * ** ***************** **** *****   *** ** ***

SmaGbpC   481 VAVPKDPGEEPVPPTEPKLTTPSVTWHKNFVVQTSKKPTPPKTTGEPPKKPTPKDPVKPT  540
SmuGbpC   458 VAVPKDPGAKPTPPEKPELKKPTVTWHKNLVVETKTEEVPPVTPPTTPDEPTPEKPKTPE  517
                ********  * **  * *  * ****** ** *     ** *    *  ***  *  *

SmaGbpC   541 PNVPVKPTPAPKSVNYSRPAIFRSAKKNEVRVRERQYTAPLPQTGDEKQYGLSVIGFVSA  600
SmuGbpC   518 ---------DPQSPVVAKSVSFRTARKGEMRVRERDYQPTLPHTGAAKQNGLATLGAIST  568
                     * *       ** * * * ***** *   ** **  ** **   *  *

SmaGbpC   601 SFAAATLMAAKRRKKEN                                            617
SmuGbpC   569 AFAAATLIAA--RKKEN                                            583
                ****** **  *****
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Fig. 3. Comparison of GbpC sequences of *S. mutans* and *S. macacae*. Computer-generated alignment of *S. mutans* and *S. macacae* GbpC proteins allowing gaps (hyphens). Identical amino acid residues between two sequences are shown as *****.

harboring pBAD/HisA expression vectors containing serially deleted partial *gbpC* homologues (pBAD-FraC, pBAD-FraD, and pBAD-FraE) were also constructed (Fig. 4). The expression of these induced His-tagged *S. macacae* GbpC homologue proteins was confirmed by CBBR staining of SDS polyacrylamide gels and Western blot analysis with anti-Xpress antibody (data not shown).

Although no positive bands were detected with the vector control (designated as pBAD) of the Western blot, positive bands with molecular masses of approximately 75, 51, 25, 44, 41, and 38 kDa were detected in the extracts of pBAD-SmaGbpC, pBAD-FraA, pBAD-FraB, pBAD-FraC, pBAD-FraD, and pBAD-FraE, respectively. This corresponded to predicted approximate molecular sizes (Fig. 4). The corresponding bands were also detected by Western blot analysis with antiserum generated against the *S. mutans* GbpC protein (data not shown). However, multiple bands were also detected, indicating that the anti-GbpC serum cross-reacted with the *S. macacae* GbpC homologue proteins.

Crude *E. coli* extracts were prepared as described in Material and methods. Glucan-binding activity was measured by immobilizing the induced proteins on nickel-coated wells in microtiter plates and measuring the binding ability of biotinylated dextran to immobilized proteins following detection with Streptavidin-horseradish peroxdase conjugate. The constructs and their dextran-binding activities are shown in Fig. 5. The vector control did not exhibit any binding to dextran. pBAD-

Glucan-binding assays

Matsumoto *et al*. (personal communication) has reported that the dextran-binding activities of *S. mutans* fragments A and B (corresponding to the 328–1607 bp and 531–1096 bp regions of the *gbpC* gene, respectively) (Fig. 4) were as high as that for the intact GbpC protein. Therefore, we constructed *E. coli* clones capable of overexpressing the full-length *S. macacae* GbpC protein homologue without a signal peptide sequence (designated as pBAD-SmaGbpC). We also constructed partial GbpC protein domains corresponding to *S. mutans* fragments A and B (designated as pBAD-FraA and pBAD-FraB). We then used these constructs to examine the glucan-binding activities of the protein products. In addition, three other clones

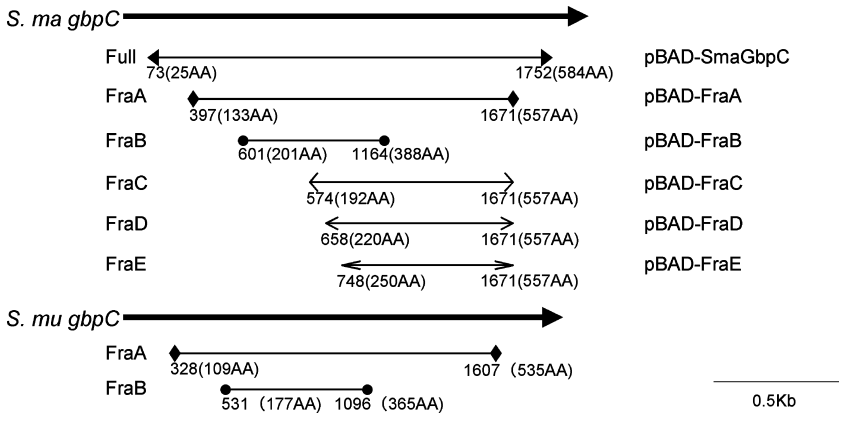

Fig. 4. Subcloning and expression of *S. macacae gbpC* homologue fragments. ◆–◆ and ●–● indicate fragment A and B regions of *S. mutans* GbpC, respectively, and homologous regions in *S. macacae* GbpC homologue (pBAD-FraA and pBAD-FraB). Other double-headed arrows indicate full length or partial length of *S. macacae* GbpC homologue (pBAD-SmaGbpC, pBAD-FraC, pBAD-FraD, and pBAD-FraE). Numbers and those suffixed with AA in parentheses indicate nucleotide and amino acid numbers at both ends of fragments, respectively.

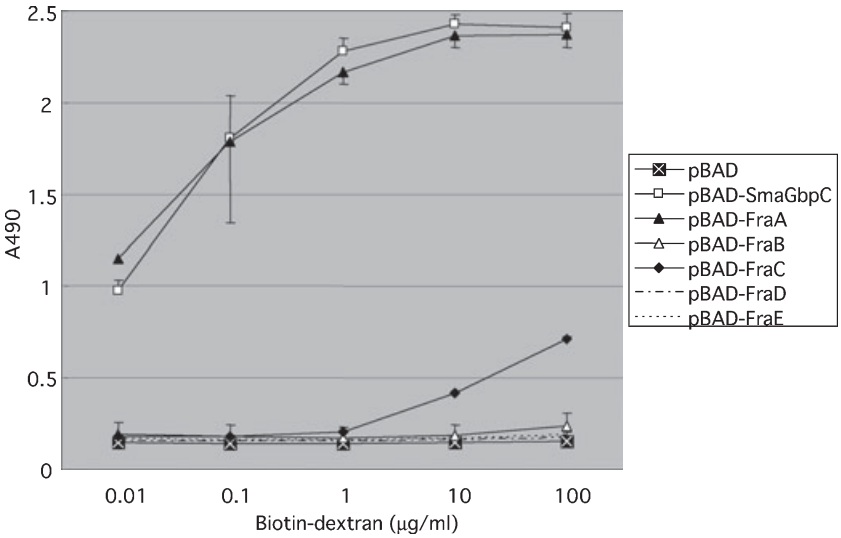

Fig. 5. Biotin-dextran binding by histidine-tagged derivatives of *S. macacae* GbpC homologue proteins. Crude extract from *E. coli* TOP10 harboring pBAD/HisA (designated pBAD) used as vector control. pBAD-SmaGbpC, pBAD-FraA, B, C, D, and E are crude extracts from *E. coli* TOP10 harboring pBAD/HisA containing fragments corresponding to those in Fig. 4. Higher A490 values equate to higher binding activities. Wells of ELISA plates were coated with equivalent amounts of protein for each extract, incubated with indicated concentrations of biotin-dextran and detected as described under Material and methods. Data presented are averages and standard errors of three independent determinations for each sample.

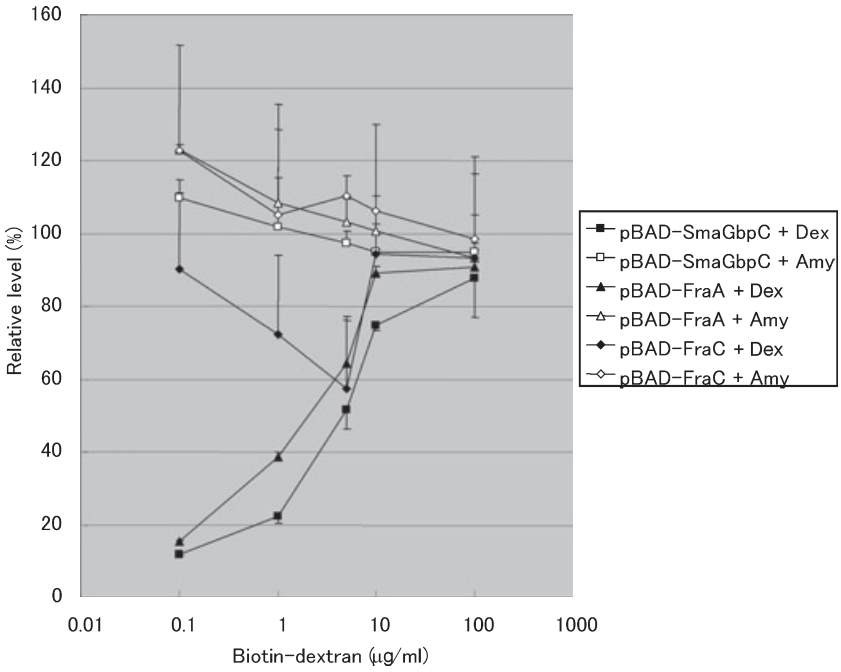

Fig. 6. Competition with dextran and amylose (α-1,4 glucan) for binding to pBAD-SmaGbpC, pBAD-FraA, and pBAD-FraC. Concentration of biotin-dextran was increased, whereas concentration of competitor was kept constant. Competitors diluted in PBS + 0.2% BSA were dextran (unlabeled dextran at 10 μg/ml) and amylose (10 μg/ml).Data are expressed as percentage of each biotin-dextran binding activity without competitor (set at 100%). Values represent averages and standard errors of three independent determinations for each sample.

SmaGbpC displayed the highest dextran-binding activity. pBAD-FraA exhibited high dextran-binding activity, similar to that of pBAD-SmaGbpC. pBAD-FraC, which codes for a partial GbpC homologue, also exhibited dextran-binding activity, although the level was low compared to that of pBAD-SmaGbpC and pBAD-FraA. pBAD-FraB, which encodes the partial GbpC homologue that is homologous to *S. mutans* GbpC fragment B, showed no dextran-binding activity, and neither did pBAD-FraD or pBAD-FraE. This suggests that the glucan-binding domain may correspond to approximately 70 amino acid residues of the N-terminus in the *S. macacae* GbpC homologue protein.

The observation that unlabeled dextran produced a reduction in signaling showed that biotin-dextran competed with unlabeled dextran (Fig. 6). The results further showed that amylose did not effectively compete with biotin-dextran for binding to immobilized pBAD-SmaGbpC, pBAD-FraA, or pBAD-FraC (Fig. 6). This suggests that α-1,4 glucan does not inhibit the binding activity of the *S. macacae* GbpC homologue. Dextran, the putative substrate for the GbpC homologue protein, competes for binding of biotin-dextran and amylose does not, at least at these concentrations. This suggests that dextran acts as a competitive inhibitor, i.e. both ligands vie for the same site on the protein.

To determine whether the *S. macacae* GbpC homologue protein had an affinity toward α-1,3 glucan, the glucan-binding activity of the diluted crude extracts of the *S. macacae* GbpC homologue protein was examined following treatment with excessive amounts of insoluble α-1,3 glucan particles. The glucan-binding activity of the protein solution treated with α-1,3 glucan showed no difference to that of the nontreated samples (data not shown).

Complementation of mutation in *S. mutans* by *in vitro* construction

The *S. macacae gbpC* homologue was introduced into ddag-negative *S. mutans* strain Z1. Complementation showed that the functions of the homologue and the *S. mutans* strain were identical. *S. mutans* strain Z1 possesses a strain-specific *cnm* gene which participates in the cold agglutination phenotype (25) and is a ddag-defective strain. These characteristics make it a suitable candidate for a host strain for transformation and subsequent screening. Twenty-six transformants were ultimately obtained and screened for cold agglutination and ddag phenotypes. Six of the 26 colonies constitutively exhibited ddag-positive and cold agglutination-negative phenotypes. In addition, nucleotide sequencing confirmed that one mutant, designated as (QV)Z1, harbored a single

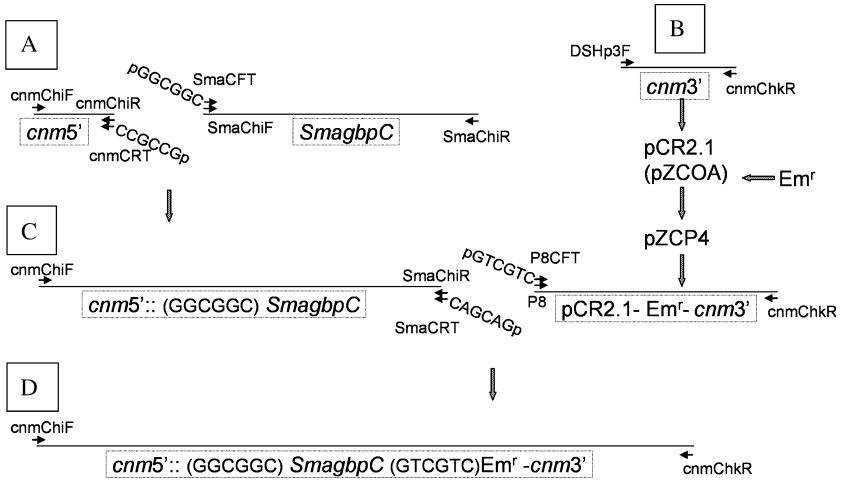

Fig. 7. Construction of fragment used in complementation test. Procedure of fragment construction described under Material and methods. Line A: strategy to construct chimeric molecule between *S. mutans cnm* 5′-gene region and fragment from *S. macacae gbpC* gene homologue. Predicted protein expected to be correctly secreted and cell wall-sorted into *S. mutans* cells. Line B: plasmid construction to align erythromycin-resistant gene upstream of partial *cnm* 3′ gene region. Line C: strategy to construct linear fragment to be introduced into *S. mutans* by allelic exchange mechanism. Line D: constructed linear fragment used to transform *S. mutans*. Following transformation of *S. mutans* with fragment, 26 colonies were detected on Mitis-Salivarius agar plates after 3 days anaerobic incubation, and transformants were screened for ddag and cold agglutination phenotype.

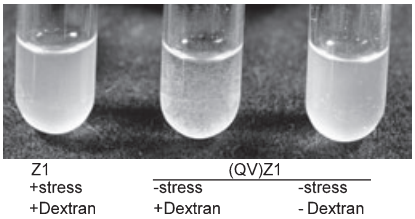

Fig. 8. Dextran-dependent aggregation of *S. mutans* Z1 transformed with chimeric molecule of *S. macacae gbpC* gene homologue. (QV)Z1 is ddag-positive and cold agglutination-negative transformant of *S. mutans* strain Z1.

nucleotide deletion in the *gbpC* gene. PCR amplification confirmed allelic exchange with the fragment constructed *in vitro* by the method described in Material and methods (Fig. 7) (data not shown). This suggests that the *S. macacae* GbpC homologue protein is a glucan aggregation protein (Fig. 8).

Southern hybridization analysis of mutans streptococcal gbpC homologues

We performed Southern hybridization analysis using the *S. macacae gbpC* homologue fragment probe with the chromosomal DNA of several mutans streptococci (*S. ferus*, *S. downei*, *S. sobrinus*, *S. ratti*, and *S. criceti*) to investigate the universality and distribution of *gbpC* homologues in mutans streptococci. Positive bands were detected in all these species, but no positive bands were exhibited in nonmutans streptococci, *S. sanguinis* strain ATCC10556, *S. gordonii* strain Challis, *S. anginosus* strain IS57, or *S. salivarius* strain ATCC9222 (Fig. 9). This suggests a broad distribution of *gbpC* homologues in mutans streptococci.

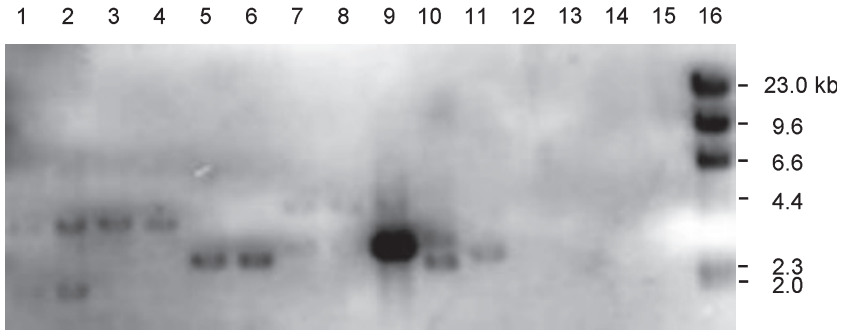

Fig. 9. Distribution of *gbpC* gene homologue in mutans streptococcal chromosomes. Chromosomal DNA digested with *Hind*III from several species of mutans streptococci were analyzed using *S. macacae gbpC* homologue fragment.lane 1: *S. criceti* HS6, lane 2: *S. criceti* OMZ61, lane 3: *S. ratti* 107P, lane 4: *S. ratti* FA-1, lane 5: *S. mutans* 109c, lane 6: *S. mutans* UA159, lane 7: *S. sobrinus* ATCC6715, lane 8: *S. sobrinus* 100–4, lane 9: *S. macacae* ATCC35911, lane 10: *S. downei* MFe28, lane 11: *S. ferus* ATCC33477, lane 12: *S. sanguinis* 10556, lane 13: *S. gordonii* Challis, lane 14: *S. anginosus* IS57, lane 15: *S. salivarius* 9222, lane 16: Size marker.

Discussion

The novelty of this study lies primarily in the detection and characterization of the glucan-binding protein C homologue in *S. macacae*. *S. sobrinus* more readily autoaggregated than did *S. mutans*, and one study has demonstrated glucan-binding lectin to be a major glucan-binding protein of *S. sobrinus* (8). Subsequently, it was reported that *S. sobrinus* possessed the glucan-binding protein capability of binding dextran, and that one of these proteins, the 60 kDa protein, appeared to be involved in ddag (15). Glucan-binding lectins have been defined as glucan-binding proteins that confer the property of aggregation on bacteria in the presence of exogenous dextran (15). Similarly, here we propose the designation Dbls (dextran-binding lectins) for a wall-anchored protein family characterized by dextran-binding activity (e.g. GbpC). This cell-wall anchoring of the protein is essential for the aggregation phenotype, as discussed below. We propose that it is reasonable to designate these proteins as Dbls, but not glucan-binding lectins, as they do not exhibit any binding activity with amylose (α-1,4-glucan) or α-1,3-glucan, as indicated in Fig. 6.

Unrooted phylogenetic trees showing the relationship between partial 16S rRNA sequences revealed a close relationship between *S. macacae* and *S. mutans* (37). Furthermore, *S. macacae* is more similar to *S. mutans* than to other mutans streptococci with respect to the serotype polysaccharide antigen, genomic GC content (5), and spontaneous generation of colonization-defective mutants (unpublished results) reported in *S. mutans* (36). This study was originally undertaken with the intent of investigating the divergent evolution of these two species. In addition, we were interested in whether *gbpC* was a widespread adherence factor in mutans streptococci. In addition to glucan-binding proteins, *S. mutans* also possesses glucan-binding glucosyltransferases (GTFs), water-insoluble glucan-synthesizing GTF-I encoded by *gtfB* (1) and *gtfC* (10) and

water-soluble glucan-synthesizing GTF-S encoded by *gtfD* (11). Glucans can also interact with Gbps to promote cell-to-cell aggregation (9, 33). The *gtf* genes and the *pac gene* (21, 22) encoding the extracellular surface *p*rotein *a*ntigen of serotype *c S. mutans* are regarded as major etiologic factors for dental caries (4, 14, 21, 22), and their homologues are reported to be widely distributed among mutans streptococci. The *gbpC* gene homologue, however, has yet to be found in species other than *S. mutans*. We expected the *S. macacae gbpC* homologue gene to exhibit a higher homology with the *S. mutans gbpC* gene because of the stringency of the Southern hybridization analysis performed (data not shown). The homology between these genes was 67% at the nucleotide level, and was not as high as expected from the results of the analysis, although the putative monocistronic organization of these two genes was the same. No ORFs were found in either strand within 730 bp upstream of the *S. macacae gbpC* ORF. In the downstream flanking sequences, the formation of a putative stem–loop structure in this region of the mRNA corresponds to a free-energy change of − 64.5 kJ/mol. In addition, anti-GbpC serum cross-reacted with the recombinant GbpC homologue protein with a 77% similarity to the GbpC protein. This was not surprising, as anti-GbpC serum contains polyclonal antibodies and these two proteins should therefore share common epitopes.

The functional domain for glucan-binding in the *S. mutans* GbpC protein has been estimated to be located within the fragment B region (Matsumoto, personal communication). However, our results for the dextran binding of the GbpC homologue do not concur with Matsumoto's reports (Fig. 5). The dextran-binding activity expressed from pBAD-FraA was as high as that from pBAD-SmaGbpC, whereas the proteins expressed from pBAD-FraB, pBAD-FraD, and pBAD-FraE did not exhibit any dextran-binding activity (Fig. 5). This difference in functional domains for dextran-binding may result from a divergence of the two homologous proteins. pBAD-FraC, which codes for a partial GbpC homologue, exhibited a lower dextran-binding activity than pBAD-SmaGbpC and pBAD-FraA. This suggests that the critical domain for glucan binding may be located near the N-terminus of the *S. macacae* GbpC homologue protein.

A *gbpC* homologue gene was detected in the *S. macacae* chromosome by PCR using primers designed from the *gbpC* nucleotide sequences of the *S. mutans* strains. We observed nucleotide polymorphisms in the *S. mutans gbpC* gene in various strains (26), and utilized these polymorphisms in designing the primers for the PCR analysis of the *S. macacae.* We also investigated whether the *S. macacae gbpC* homologue gene was a divergent molecular species. Initially, we attempted to detect *gbpC* homologues from *S. macacae* by colony and plaque hybridization. However, we found no positive clones or plaques, even after screening a number of clones corresponding to more than a 100-fold coverage of the entire genome.

As *S. macacae,* like *S. sobrinus* (6), is neither naturally nor artificially transformable (unpublished result), constructing a knock-out mutant in this species is difficult. Therefore, in this study, we attempted to demonstrate complementation using chimeric fragments constructed *in vitro* by PCR, and the results were successful (Fig. 8). Accordingly, we believe that the *S. macacae gbpC* homologue gene is responsible for the ddag phenotype of this organism. It is highly likely that the *gbpC* homologue gene is an important factor in the ddag of *S. macacae,* since the GbpC homologue protein appears to be a wall-anchored protein and exhibits dextran-binding activity (Fig. 5). The GbpC homologue protein is a member of the wall-anchored protein family and possesses a potential anchor domain consisting of four characteristic features:

- a hydrophilic wall-spanning region rich in glycine and proline;
- an LPxTG motif, which is the consensus sequence for gram-positive cocci surface proteins;
- a hydrophobic membrane-spanning region;
- a cytoplasmic charged tail in its C-terminal region (19).

Igarashi *et al*. (12) demonstrated that an *S. mutans* sortase enzyme encoded by the *srt* gene mediated the cell wall-anchoring of the GbpC protein, and that an *srt* mutant of this organism had lost the ddag phenotype. Therefore, a cell wall-anchored GbpC homologue protein with glucan-binding capability is the most likely candidate for the ddag phenotype of *S. macacae*.

S. macacae exhibits the ddag phenotype constitutively, as do *S. sobrinus* and *S. criceti*, whereas *S. mutans* exhibits inducible ddag only after growth under stress conditions, i.e. cells grown at 42°C or growth with subinhibitory concentrations (0.18 μg/ml) of tet. However, the extent of aggregation in *S. macacae* is more similar to that in *S. mutans* than to that in *S. sobrinus* (Fig. 2). In this respect, *S. macacae* exhibits an intermediate aggregation phenotype among these mutans streptococci. Moreover, only *S. macacae* and *S. mutans* exhibited positive bands when probed with the *S. mutans gbpC* gene fragment; the serotype *a–h* mutans streptococci exhibited positive bands when probed with the *S. macacae gbpC* homologue fragment under Southern hybridization analysis (Fig. 9). These results suggest that the *S. macacae gbpC* homologue gene is closer to ancestral sequences than the other *gbpC* homologues in mutans streptococci. This may be compatible with the intermediate aggregation phenotype of *S. macacae*. In early investigations, glucan-binding proteins involved in ddag were regarded to be specific for *S. sobrinus* and *S. criceti* (8). However, we found that the *gbpC* gene was involved in the *S. mutans* ddag. Moreover, we also detected the *gbpC* homologue in *S. macacae*. Positive Southern hybridization bands were detected even in *S. ferus* (Fig. 9), which was reported to exhibit only 1.0% hybridization with *S. macacae* in DNA base-pairing experiments (37). Recently, it has been questioned whether *S. ferus* should truly be regarded as a mutans streptococcus (2, 24, 37). Although the classification of *S. ferus* still needs further consideration, we detected positive Southern hybridization bands in this species. The widespread occurrence of *gbpC* gene homologues in mutans streptococci observed in this study suggests that they may play an important role in the survival of mutans streptococci in plaque biofilms. It is probable that *gbpC* homologues may also be later identified in other species of mutans streptococci such as *S. sobrinus* and *S. criceti*. A homology search against the TIGR Microbial database for the unfinished *S. sobrinus* sequence showed five gene regions with relatively high homology scores for the *S. mutans* GbpC protein sequence. Therefore, a positive identification of the actual *gbpC* gene homologue cannot be made at present. We have not yet obtained positive results for *S. sobrinus* with the primer sets used in this work. We are currently examining the conditions and designing the primers necessary to detect *gbpC* homologues from other mutans streptococci by PCR analysis for subsequent isolation and characterization.

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