

Cloning and Sequence Analysis of the *gbpC* Gene Encoding a Novel Glucan-Binding Protein of *Streptococcus mutans*

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We have isolated dextran-aggregation-negative mutants of *Streptococcus mutans* following random mutagenesis with plasmid pVA891 clone banks. A chromosomal region responsible for this phenotype was characterized in one of the mutants. A 2.2-kb fragment from the region was cloned in *Escherichia coli* and sequenced. A gene specifying a putative protein of 583 amino acid residues with a calculated molecular weight of 63,478 was identified. The amino acid sequence deduced from the gene exhibited no similarity to the previously identified *S. mutans* 74-kDa glucan-binding protein or to glucan-binding domains of glucosyltransferases but exhibited similarity to surface protein antigen (Spa)-family proteins from streptococci. Extract from an *E. coli* clone of the gene exhibited glucan-binding activity. Therefore, the gene encoded a novel glucan-binding protein.

Mutans streptococci have been implicated as the most important microbial agents in dental caries (12), and one of the recognized virulence properties of these organisms is their ability to adhere to tooth surfaces in the presence of dietary sucrose. The roles and mechanisms of glucosyltransferases (GTFs) in adhesion to a variety of surfaces have been investigated by genetic approaches, and many of the genes specifying GTFs have been cloned from streptococci during the past decade (9). These organisms also produce multiple glucan-binding proteins (GBPs) (13, 15), which presumably promote the adhesion of the organisms. Although an 87-kDa GBP (31) from *Streptococcus sobrinus* and 74-kDa (20) and 59-kDa (27) GBPs from *Streptococcus mutans* have been characterized, until now only one GBP (74-kDa Gbp) has been cloned and sequenced (2).

It has been reported (3, 6) that *S. sobrinus* and *Streptococcus cricetus* exhibit rapid aggregation of cell suspensions upon addition of dextran T2000, while *S. mutans* serotype c, e, and f strains fail to do so. Although some GBPs should be involved in this aggregation, no genes responsible for this phenotype in *S. sobrinus* or *S. cricetus* have been identified so far. Subinhibitory concentrations of some antibiotics were reported to have enhanced the dextran-dependent aggregation of *S. sobrinus* (30). We have found that some of the *S. mutans* serotype c and e strains grown with a subinhibitory concentration of tetracycline exhibited rapid aggregation of the cells upon addition of dextran T2000.

In order to define a protein involved in this dextran-dependent aggregation of *S. mutans* based upon a genetic analysis, we constructed random mutants in a transformable *S. mutans* strain by using pVA891 clone banks and isolated dextran-aggregation-negative mutants of *S. mutans*. A chromosomal region responsible for this phenotype was characterized in one of the mutants, and the corresponding gene was cloned in *Escherichia coli* by a marker rescue method. Nucleotide sequence analysis of the gene suggested that the gene encoded a novel GBP associated with the cell surface, and thus we designate this gene *gbpC*. We describe in this communication the cloning, sequencing, and characterization of the gene.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *S. mutans* 109c (serotype c), its spontaneous colonization-defective strain 109cS, and *E. coli* HB101 and JM109 were maintained and routinely grown as previously described (32). *S. mutans* strains and plasmids used in this study are described in Table 1.

Random mutagenesis of *S. mutans* and screening of the mutants. To randomly mutagenize the *S. mutans* chromosome, pVA891 clone banks were utilized as described previously (24, 32). The clone banks were constructed with *Bam*HI-digested plasmid pVA891 (14) and completely *Sau*3AI-digested chromosomal DNA fragments of *S. mutans* 109cS.

All of the transformants detected on Todd-Hewitt agar plates following transformation of strain 109cS with the clone banks were inoculated into 150 μ l of BTR broth (25) containing 0.4% glucose (BTR-G) and 0.18- μ g/ml tetracycline (BTR-G/tet), which was then dispensed into 96-well microplates. The microplates were sealed with adhesive seal sheets (MS-30010; Sumitomo Bakelite Co. Ltd., Tokyo) to prevent evaporation of medium and incubated overnight at 37°C without shaking. Cells were suspended by pipetting, and a solution of dextran T2000 (molecular weight, 2,000,000; Sigma, St. Louis, Mo.) was added to each well to a final concentration of 100 μ g/ml. Microplates were then subjected to shaking with a micromixer (Taitec EM-36; Koshigaya City, Saitama, Japan) for 5 to 10 min at a low to medium range of shaking, the suspensions were observed on a specially devised light box for aggregation, and nonaggregated clones were initially selected as presumable dextran-dependent aggregation-negative (ddag⁻) mutants.

For the secondary screening, each of the initially isolated mutants was inoculated into three screw-capped glass tubes each containing 2 ml of BTR-G broth, and the tubes were incubated overnight under three different conditions, i.e., incubated with either 0.18 μ g of tetracycline/ml or 4% ethanol or incubated at 42°C. Each overnight culture was divided into two 1-ml portions, and dextran T2000 (100 μ g/ml) was added to one of them. After swirling for a few minutes, each pair of tubes was observed for aggregation by the unaided eye. Clones exhibiting no visible differences between the pair of tubes from any three incubations were tentatively identified as the ddag⁻ mutants and stocked frozen at -80°C.

Southern hybridization analysis of ddag⁻ mutant chromosomes. Appropriate restriction enzyme-digested chromosomal DNA fragments from ddag⁻ mutants were separated following agarose gel electrophoresis and transferred to nylon membranes (NytranN; Schleicher and Schuell, Dassel, Germany) as described previously (21, 24). The fragments were analyzed by the enhanced chemiluminescence (ECL) direct nucleic acid labelling and detection system as recommended by the supplier (Amersham Co. Ltd., Tokyo).

Recovery of DNA fragments flanking the pVA891 insertion. Following Southern hybridization analysis of chromosomal DNA of the ddag⁻ mutants, flanking regions containing plasmid pVA891 were recovered in *E. coli* HB101 as previously described (32). The recovered fragments flanking pVA891 were extensively characterized following restriction endonuclease digestion with enzymes selected on the basis of the Southern blot hybridization patterns of this region of the chromosome.

Nucleotide sequencing and sequence analysis. DNA fragments to be sequenced were subcloned into pBluescript KS+/SK+ (Stratagene) or pKmOZ'18/19 (26). Preparation of sequential deletion clones by using the exonuclease III and mungbean nuclease system (Stratagene) and sequencing with *Taq* cycle sequencing kits (PE Applied Biosystems and Amersham Co. Ltd.) and an automated DNA sequencer (PE Applied Biosystems) were accomplished as described pre-

TABLE 1. *S. mutans* strains and plasmids

Strain or plasmid	Description ^a	Source or reference
<i>S. mutans</i>		
109c	ddag ⁺ , a clinically isolated transformable strain	This study
109cS	ddag ⁺ , a spontaneous colonization defective mutant of 109c due to the homologous recombination between the <i>gfb</i> and <i>gfc</i> genes	This study
32A02	ddag ⁻ , a transformant of 109cS isolated following random mutagenesis	This study
LSMD91	ddag ⁺ , a deletion mutant of 109cS; <i>EcoRV</i> region depicted in Fig. 2 was deleted from chromosome and replaced with the Em ^r gene fragment	This study
LSLA91	ddag ⁺ , a deletion mutant of 109cS; <i>Clal-BstYI</i> region depicted in Fig. 2 was deleted from chromosome and replaced with the Em ^r gene fragment	This study
LSM191	ddag ⁻ , a deletion mutant of 109cS; <i>XbaI-PstI</i> region depicted in Fig. 2 was deleted from chromosome and replaced with the Em ^r gene fragment	This study
LSMF91	ddag ⁻ , a deletion mutant of 109cS; <i>HindIII-BstYI</i> region depicted in Fig. 2 was deleted from chromosome and replaced with the Em ^r gene fragment	This study
SM591	ddag ⁻ , an insertion mutant of 109cS; the Em ^r gene and plasmid pKmOZ'19 was inserted into chromosome as depicted in Fig. 2	This study
LSSL91	ddag ⁺ , a deletion mutant of 109cS; <i>XbaI-AseI</i> region depicted in Fig. 3B was replaced with the Em ^r gene in the same direction with the <i>gbc</i> gene	This study
LSLM91	ddag ⁻ , the same as LSSL91 except that the orientation of the Em ^r gene was reverse	This study
Plasmid		
pVA891	A marker rescue plasmid containing the Em ^r gene used to randomly mutagenize <i>S. mutans</i> chromosome	14
pBluescript KS+/SK+	Phagemid cloning vector	Stratagene
pKmOZ'18/19	Km ^r -pUC type vector	26
pGEM3zf+	Used as T7 promoter vector in this study	Promega
pGP1-2	A plasmid carrying the T7 RNA polymerase gene	28
pSLC1	The <i>gbc</i> gene was subcloned into pGEM3zf in the same direction as the T7 promoter	This study
pSLU7	5' region of the <i>gbc</i> gene was removed from pSLC1	This study

^a ddag⁺, dextran-dependent aggregation positive; Km^r, kanamycin resistance.

viously (32). The nucleotide sequences of both strands of the 2.2-kb *EcoRV-ClalI* fragment were determined. Sequence analysis was carried out with the DNASIS-Mac program (Hitachi Software Engineering). The international DNA databases (EMBL, GenBank, and DDBJ) were searched for similar amino acid sequences by using the FASTA program.

Construction of deletion and insertion mutants in *S. mutans*. Deletion and insertion mutants were respectively constructed by allelic exchange and by integration of plasmids containing an internal fragment of the target gene as described previously (32). The predicted deletion of the fragment from or insertion of the plasmid into the chromosome of *S. mutans* was confirmed by Southern hybridization analysis.

Expression of the *gbc* gene with a T7 RNA polymerase/promoter system. The *gbc* gene was subcloned into plasmid pGEM3zf (Promega, Madison, Wis.) in the opposite direction relative to the *lac* promoter (in the same direction as the T7 promoter). The resultant plasmid, pSLC1, was then introduced into *E. coli* JM109 harboring the plasmid pGP1-2 coding for the T7 RNA polymerase and expressed according to the previously described procedure (28). As a negative control, about 80% of the 5' region (*EcoRV-BamHI*) of the *gbc* gene was removed from pSLC1, and the resultant plasmid, pSLU7, was also introduced into the same host. The cells were harvested, washed once with 10 mM potassium phosphate buffer (pH 6.5), and resuspended in 2 ml of the same buffer.

Preparation of crude extracts. *E. coli* cells were grown and suspended as described above. *S. mutans* cells were grown overnight at 37°C in 10 ml of the BTR-G medium, and the cells were collected, washed once, and resuspended in the same buffer at a concentration of 0.2 g (wet weight)/ml. Both *E. coli* and *S. mutans* suspensions were subjected to mechanical disruption with glass beads in a B. Braun MSK cell homogenizer for 5 min and 15 min, respectively in the cold room (4°C). After the removal of the glass beads and cell debris by low-speed centrifugation, the extract was microcentrifuged at 12,000 rpm (10,000 × g) for 15 min, and the supernatant was stored at -20°C as a crude extract until use. A portion of the extract was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, and an aliquot was used for the glucan-binding assays.

Glucan-binding assay. Glucan-binding assays were carried out by use of biotin-dextran (molecular weight, 70,000; Sigma) as described previously (11) except for the following modifications. For this assay, the wells of enzyme-linked immunosorbent assay (ELISA) plates (Iwaki Glass, Funabashi, Japan) were coated with 50 μl of *E. coli* crude extracts (0.7 mg of protein per ml) for 18 h at 4°C. *o*-Phenylenediamine dihydrochloride was used as a substrate of the color detection solution, and the resulting *A*₄₉₀ after incubation with the detection solution for 1.5 min was measured in a microplate reader (Bio-Rad).

SDS-PAGE. A portion of the extract was mixed with the SDS sample buffer and frozen until electrophoretic analysis was performed using the gel system of Laemmli with 7% acrylamide gels. Samples were boiled for 3 min immediately before being applied to the gels. The molecular mass of the protein encoded by the *gbc* gene was determined following electrophoresis and staining of the gels with 0.1% Coomassie brilliant blue R.

Western blot analysis. Western blot analysis was carried out as previously described but with a slight modification (29). Following SDS-PAGE, the separated proteins were transferred to a supported nitrocellulose membrane, Hybond-C super (Amersham), by a semi-dry electrotransfer apparatus (Model AE6675; ATTO Corp., Tokyo). The nitrocellulose membrane was preincubated with Tris-buffered saline containing 5% bovine serum albumin for 1 h at 37°C to block nonspecific protein binding. The primary antibodies were generated with antiserum either against the *S. mutans* surface protein antigen (Spa) PAc (protein antigen of serotype C [16]) or against the *S. mutans* 59-kDa GBP, GBP59 (27) (kindly supplied by T. Koga, Kyushu University and D. J. Smith, Forsyth Dental Center, respectively), and the secondary antibody was goat immunoglobulin G conjugated to horseradish peroxidase (Organon Teknica Corp., West Chester, Pa.). The ECL Western blotting detection reagent (Amersham) was employed for the detection.

Nucleotide sequence accession number. The *gbc* nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number D85031.

RESULTS

Dextran-dependent aggregation of *S. mutans* cells. We have found that some *S. mutans* serotype c and e strains grown with a subinhibitory concentration of tetracycline exhibit rapid aggregation of cells upon addition of 100 μg of dextran T2000/ml, although it has been reported that *S. mutans* serotype c, e, and f strains do not aggregate in the presence of dextran T2000 (3, 6). Moreover, similar aggregation could be observed upon addition of dextran into cultures grown under a variety of conditions, including growth at 42°C incubation or growth with 4% ethanol, 80 μg of nalidixic acid per ml, 400 μg of canavanine (nonmetabolizable amino acid analog) per ml, 62.5 μg of spec-

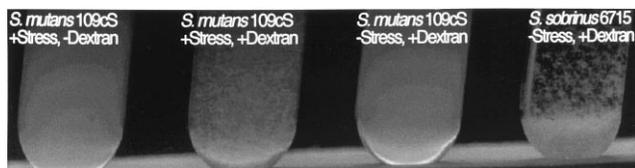


FIG. 1. Dextran-dependent aggregation of *S. sobrinus* 6715 and *S. mutans* 109cS with (+stress) or without (–stress) 0.18 μ g of tetracycline/ml.

tinomycin per ml, or 1% ammonium sulfate (data not shown). Most of the reagents added to the medium are known to induce a stress reaction for a variety of cells. Therefore, we tentatively defined these growth conditions as stress conditions. Figure 1 shows the appearance of dextran-dependent aggregated cells of *S. sobrinus* 6715 and *S. mutans* 109cS grown in BTR-G broth (the composition of which was modified in our lab [25]) with (+stress) or without (–stress) 0.18 μ g of tetracycline/ml, and aggregation was never observed without dextran T2000. The aggregation of *S. mutans* 109cS was not as strong as that of *S. sobrinus* 6715, as shown in Fig. 1, but it was distinctively visible. The aggregation was competitively inhibited by low-molecular-weight glucan (Dextran T10) and completely inhibited by addition of 1 mM EDTA to the culture. The aggregation was considerably dependent on the growth medium and could not be observed or was difficult to detect with brain heart infusion broth or Todd Hewitt broth. The mechanisms of this dependency on the medium are not known at present. All of the factors described above may have concealed *S. mutans* dextran-dependent aggregation and suggest that aggregation may be controlled by several factors.

Isolation and characterization of dextran-dependent aggregation-negative mutants of *S. mutans*. Construction of a marker rescue plasmid clone bank and random mutagenesis of the *S. mutans* 109cS chromosome were carried out as described in Materials and Methods. Approximately 7,000 colonies were totally screened for the initial step with 10 transformations (about 700 transformants were obtained per transformation procedure), and 69 possible *ddag*[–] mutants were selected. These mutants were then secondarily screened as described in Materials and Methods, and 44 *ddag*[–] mutants were tentatively identified.

*Eco*RI- and *Hind*III-digested chromosomal DNA fragments from the 44 *ddag*[–] mutants were analyzed by probing the Southern hybridization patterns with labeled plasmid pVA891 (data not shown). Since a unique *Eco*RI site of pVA891 should be located near the middle of the plasmid when integrated, two bands should be detected in the lanes to which *Eco*RI-digested chromosomal DNAs from the mutants were applied. Meanwhile, only one band is usually detected following the *Hind*III digestion, because the unique *Hind*III site on pVA891 should be at one end of the integrated pVA891. When the hybridization patterns of the chromosomal DNA from the 44 *ddag*[–] mutants digested with these two enzymes were compared, all of them were different except for those from three mutants. Further comparison of the patterns of *Pst*I- and *Xba*I-digested chromosomal DNAs from the mutants suggested that plasmid pVA891 integrated to the same site on the chromosome in these three mutants. Therefore, one of the mutants, 32A02, was further characterized.

To confirm the genetic linkage of the pVA891 insertion with an alteration of the phenotype in the mutant 32A02, chromosomal DNA from 32A02 was transformed into parental strain 109cS. Several of the transformants examined were all negative in dextran-dependent aggregation. Southern hybridiza-

tion analysis (data not shown) of the chromosomal DNA of 32A02 and 109cS suggested that a deletion may have occurred in a region flanking the pVA891 on the 32A02 chromosome. In order to recover a deleted region in 32A02, additional flanking regions were obtained from the chromosome of 109cS by chromosomal walking from both ends of the deleted region by using the marker rescue method.

Several deletion and insertion mutants were constructed with the recovered fragments, as described in Materials and Methods. Restriction maps of the fragments used to construct those mutants are indicated with the corresponding chromosomal regions of mutant 32A02 and its parental strain 109cS in Fig. 2. These results suggested that a gene region responsible for dextran-dependent aggregation encompasses a 1.3-kb *Pst*I-*Hind*III fragment within the 2.2-kb *Eco*RV-*Cla*I fragment indicated in Fig. 2.

Nucleotide and deduced amino acid sequences of the *gbc* gene. The nucleotide sequence of the 2.2-kb *Eco*RV-*Cla*I fragment was determined as described in Materials and Methods. The 2,195-bp nucleotide sequence shown in Fig. 3A begins at an *Eco*RV site and ends at a *Cla*I site. The 1,752-bp open reading frame (ORF) encompasses residues 241 to 1992, beginning with an ATG and terminating with a TAG codon. This ORF would encode a 583-amino-acid protein with a calculated molecular weight of 63,478. A potential ribosome-binding site (AGGA) could be identified 8 bp upstream from the ATG initiation codon of the *gbc* gene. In addition, a promoter-like sequence (TTGAAA-N₁₇-TATAAT), which resembles the *E. coli* promoter consensus sequence, exists between residue 168 and 196, situated 45 bp upstream from the initiation codon of the *gbc* gene. An inverted repeat sequence characteristic of transcription terminators could also be detected between residues 2072 and 2112, situated 79 bp downstream from the termination codon of the *gbc* gene. The formation of a putative stem-loop structure in this region of the mRNA corresponds to a free energy change of –115.1 kJ/mol. These results suggest that the *gbc* gene from *S. mutans* is monocistronic. Since a restriction enzyme cleavage site (*Ase*I) was found immediately upstream from the Shine-Dalgarno sequence of the *gbc* gene (Fig. 3A), we constructed two mutants of *S. mutans*, LSL91 and LSLM91, in which the putative promoter regions upstream from the *Ase*I site were removed from the chromosome following replacement with the erythromycin resistance gene, *Em*^r (see restriction maps in Fig. 3B). These two mutants were isogenic but the direction of the *Em*^r genes inserted into the chromosome were opposite to each other. LSL91 exhibited dextran-dependent aggregation similar to the parental strain, even though the *gbc* gene was not expressed from its own physiological promoter but was apparently expressed by read-through transcription from the *Em*^r gene, while LSLM91 was *ddag*[–]. These results suggest that regulation of the dextran-dependent aggregation of *S. mutans* may be controlled not only by expression of the *gbc* gene but also by other unknown factors as will be discussed below.

The *gbc* gene product contained several features generally found in surface proteins of gram-positive bacteria. A Kyte and Doolittle hydrophobicity plot (data not shown) of the deduced protein indicated two apparent hydrophobic stretches at its amino and carboxy termini, and the average value of the whole sequence was calculated to be –0.72. The amino terminus codes a signal peptide-like sequence, although it is not as typical as other streptococcal signal peptides relative to the high content of basic amino acids in the latter. This putative signal peptide may be 39 amino acids long, because of its potential signal peptidase site, and the calculated molecular mass of the putative mature protein should be 59 kDa, which

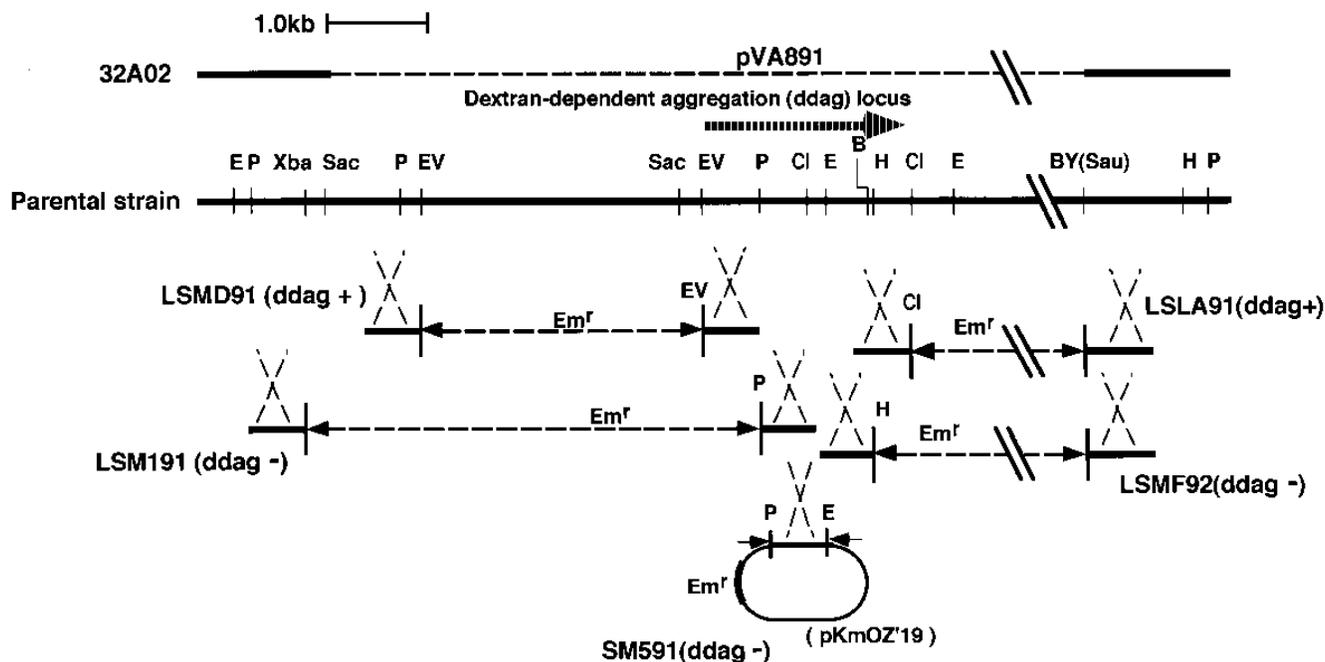


FIG. 2. Restriction map of chromosomal DNA in the mutant 32A02 and parental strain 109cS and the fragments used for construction of deletion and insertion mutants to determine the region responsible for the $ddag^-$ phenotype. In the deletion mutants, the fragments depicted by broken arrows were deleted and replaced by the Em^r gene. Following transformation of 109cS with the linearized plasmids, individual mutants were constructed by double crossover recombination mediated through the homologous regions (depicted by thick lines) which have been subcloned into these plasmids together with the Em^r gene. To construct an insertion mutant, the fragment depicted by the opposing arrows was subcloned into pKmOZ'19 together with the Em^r gene, and the resultant plasmid was then utilized to transform 109cS. The plasmid was integrated into the chromosome by a single recombination event mediated through the homologous fragment. Abbreviations: $ddag^+$, dextran-dependent aggregation positive; B, *Bam*HI; BY, *Bst*YI; Cl, *Cl*aI; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; P, *Pst*I; Sac, *Sac*I; Sau, *Sau*3AI; Xba, *Xba*I.

is interestingly similar to that of the GBPs recently purified independently by two groups from *S. mutans* (27) and *S. sobrinus* 6715 (13). A consensus sequence for gram-positive cocci surface proteins (L-P-x-T-G-[STGAVDE]) was found at residue 549 of the protein, and the carboxy-terminal sequence (-R-K-K-E-N) was very similar to the membrane anchor region of other surface proteins from gram-positive cocci (8). A possible cell wall-associated region was also found between amino acid residues 497 and 521 and contained 11 proline residues distributed regularly.

Another feature of the overall amino acid composition of the putative mature GbpC protein was high alanine (10.8%) and proline (6.1%) contents, which were similar to those (12.1 and 4.9%, respectively) of the 58- to 60-kDa glucan-binding lectin (GBL) recently isolated from *S. sobrinus* by Ma et al. (13).

The international DNA databases were searched for similar amino acid sequences with the amino acid sequence deduced from the *gbc* gene. Similar sequences were found in a Spa family of proteins from streptococci, e.g., SpaP, PAC, SpaA, and SSP5, although the molecular sizes of these proteins were obviously different from that of the GbpC protein. The extent of similarities of the corresponding regions between the query sequence and those target sequences ranged from 20.5 to 28.5%. The PAC sequence (18) offered the highest initial score by the FASTA search, while the SSP5 sequence (4) yielded the highest optimized score. The GbpC protein has no similarity to the 74-kDa Gbp of *S. mutans* or the carboxy-terminal repeating domains involved in glucan binding of glucosyltransferases from mutans streptococci. The sequences of the GbpC protein and the PAC protein of *S. mutans* (serotype c) were partially aligned and are shown in Fig. 4. Although the corresponding

region of the PAC protein contains the latter half of the second alanine-rich repeat and the third alanine-rich repeat, such a repeating unit was not detected in the GbpC protein by homology dot matrix.

Expression of the *gbc* gene in *E. coli* and glucan-binding assays. Since the intact *gbc* gene could not be subcloned into high-copy-number plasmids, e.g., pBluescript KS+/SK+, in the same direction as the *lac* promoter, a T7 RNA polymerase/promoter system was employed to overexpress the *gbc* gene in *E. coli* as described in Materials and Methods. When crude extracts from the *E. coli gbc* clone (pSLC1/pGP1-2) and the control strain (pSLU7/pGP1-2) were examined for glucan binding, it was obvious that the GbpC protein bound glucans, as shown in Fig. 5.

Western blot analysis. Crude extracts from respective strains were subjected to SDS-PAGE. A Coomassie blue-stained gel and a Western blot with antiserum for the PAC protein are presented in Fig. 6. Positive bands with molecular masses of 70, 73, 76, 81, and 95 kDa were detected in lane 3 of the Western blot, which contained the extract of the *E. coli gbc* clone (pSLC1/pGP1-2) (Fig. 6 [left]); corresponding bands were also detected with the same sample in the Coomassie blue-stained gel. Meanwhile, no positive bands were detected in the control lane (lane 2, pSLU7/pGP1-2) of the Western blot. This means that anti-PAC serum cross-reacted with the GbpC protein. Positive bands detected in a higher-molecular-weight region with an *S. mutans* extract as a positive control (Fig. 6, lane 1) likely represent the PAC protein and its degradation products. However, a positive band corresponding to GbpC protein in the same lane was not detected probably because of low cross-reactivity.

Since our cloned *gbc* gene encoded a 59-kDa putative

A

EcoRV

1 GATATCAGT¹TTAGACTCTATTTT¹⁰⁰TTTGGTAAAAATTCGGATT²⁰⁰TTAAAACAGAAAATTCAAAAATAAGGATANTAGATAAGAGAAAGCACTTTGGTTCAAT

101 TGGAACTATTTTAAAGTGAATAATTTGTAATAAAAAAGACATATTTT³⁰⁰TTAATTTATGAGTCTTATTC⁴⁰⁰TTGAAAAATGTCTTTTATTGTTATAAT⁵⁰⁰TAAA

AseI

201 ATTGTATTC¹TTATGAATTAATAATAATAAAGGATGGT²⁰TTTATGAAATCGAAAAC⁴⁰TGCTAAAAT⁶⁰TACTTTGCTAAGCAGCCT⁸⁰TTGGCGGCTTTTGGGA

S.D. *gbc*...M K S K T A K I T L L S S L A L A A F G

301 GCAACGAAT¹TTTTCAGATGAAGCATCAACTCAATTAAT²⁰TCTGTACTGTTGAGCTCC⁴⁰TACTGTGATACACAAGCATCAGAACC⁶⁰GGCTGCAACAG

401 AAAAAGAAG¹CTCTCTCTGTTAAGTCTGTTGCAAA²⁰AGTCCACACACAAGGAATACACAACGACAACAT⁴⁰CTCAAGTTACTTTCTAAAGAA⁶⁰TTGGAAGATGC

PstI

501 TAAGGCTAAT¹GCATAACAGGAAGGCTTGAAGTCACT²⁰GAAACTGAAACTCAAAAACAGCCT⁴⁰TCGGTGAAGCTGCAGATGCAGATAACAAGCACAGGCA

601 CAAACAAT¹TAATACAGCGGTAGCTGATTTATCAA²⁰AGGCAAAAGCTGAATTTCCCTCAAAAACAAGAACATATAATAAGAT⁴⁰TTTNGAAAAGTATCAGTCTG

701 ATGTCAAGG¹AGTATGAAGCTCAAAAAGGAGCCT²⁰TACGAGCAATATAAAAAAGAACT⁴⁰TGCACAAGGTTGGCATCTGGGCGT⁶⁰TTGAAAAGCCCAAGGACT

801 TGTCTTAT¹TAATGAACCTGAGGCAAAAAC²⁰TTTCTATTTGAGGGTGTTAATCAGTACCTAACA⁴⁰AAAAGAACGACGCTCAAAAACATGCAACTGAAGATATCTCT

901 CAGCAATATA¹TACTGATAATTTATACAGCTGCTG²⁰ATTTTATCCCAAGTAAATCCATATGATCCAAAAGAAATAC⁴⁰TTGGTTCAAAATGAAAGTAGCAGATC

1001 AGATTTCAG¹TACCTATGATAATATCGTTAATTTCAA²⁰AAATATAATGATAAAAAAGAT⁴⁰TAGTAAGGTAAAGATTAATATACTCTCAATAGT⁶⁰TCAACGAATAA

1101 CGAAGGCA¹GCTGCTCAATTTGTTCCATGATCCAAC²⁰TAAGACAATTTTCAATGGTGCACAGACATCTAATGCTGGCAGAAATGATAAAATCAGTGTG

ClaI

1201 ACGAATGCA¹AAATTTATTTATGATGAAAATGGCA²⁰ATGAAATTCGATTTAAGCAGCAATAATGCCATATGAGTCTCTCATCGTTAAACCAITGGGAGACTA

EcoRI

1301 AGTATGGC¹GATCATGTGAAAAAGTAAACCTTGGG²⁰GATAATGAATTCGTTAAAATACCGGGCTCATCTGTTGACTTACATGGCAATGAAATCTATTGGC

1401 TAAGGACA¹ACCAATATAAAGCTAATGGTGAACCT²⁰TTAATGGTGTAGGAGCAGATGGCTGGATGCTGCAATGCTGATGGAACCCACCTGCTGCCAGG

1501 GCTTATTT¹GGTGGCAGGTGCTATGACTTACAAGGG²⁰AGAACCCCTCACCTTTACTGTTGGTGTAAATGATCAAAAAC⁴⁰TACCAACAACCAITTTGGTTTCCGA

1601 CTAATTCAG¹CTGTAGCTGTGCCATAAAGATCCGG²⁰GAGCTAAACCAACCCGCCAGAAAACAGAGTTGAAAAACCTACTGTGACTTTGGCATAAAAATCT

BamHI

1701 TGTGTGTA¹AACTAAACTGAGGAAGTCCCTCCAGT²⁰GACACCACCAACAACCTCCTGATGAACCAACCGCAGAAAACCAAAAACACCAGAGGATCTCTCAA

Hin dIII

1801 TCACCTGT¹CTAGCTAAGTCAAGCTTTAGAACAGCA²⁰AGAAAAGGAGAAATGCGTGTAGAGAGCGTATTATCAACCGACTCTTCCACATCTGGGG

1901 CTGCTAA¹CAAAAATGGTTAGCTACTCTTGGTGTCT²⁰TTCAACTGCATTTGCTGCGGCTACTTTGATTGCAGCTAGAAAAAAGAAAAC⁴⁰TAGAGTGTAA

2001 TTCTATA¹AAAAGAGGTTGGGACAAAAAAGTGT²⁰CCGCCCTTTTATTATGTATAGTAGTTGATFAGATFAGACTCAAGCCCTTAAACAGTCTACTGAACTGT

ClaI

2101 TGAAGGCT¹GGAGCAGCACTCTTGTGTAATACCTAG²⁰TCAATTTTCCGGAGGCTGGCAGCAGAAATGGAATCACAATTTCAATACGATCTATCGAT

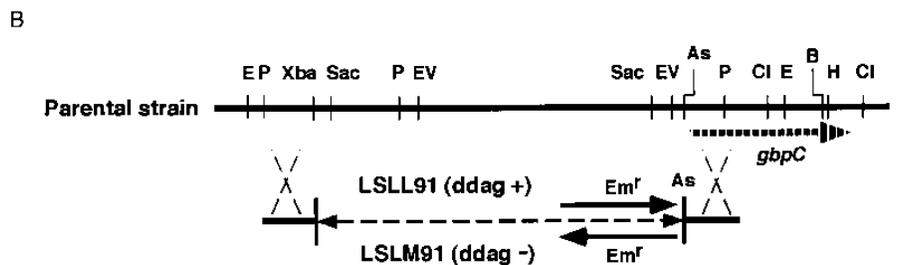


FIG. 3. (A) Nucleotide sequence and deduced amino acid sequence of the *gbc* gene region. Numbering begins at the *EcoRV* site and the sequence ends at the *ClaI* site 203 bp downstream from the termination codon of the *gbc* gene. The deduced amino acid sequences specified by the ORFs are presented below the nucleotide sequence. The opposing arrows downstream from the *gbc* gene denote an inverted repeat. The boxed sequence is a consensus sequence for gram-positive cocci surface proteins (L-P-x-T-G-[STGAVDE]). The nucleotide sequences marked -35 and -10 denote a putative promoter sequence of the *gbc* gene. A putative ribosome binding site is indicated by S.D. (Shine-Dalgarno). (B) Construction of promoter-deletion mutants. Putative promoter regions upstream from the *AseI* site were removed from their chromosome by replacement with the *Emr* gene oriented in both directions. As, *AseI*. Other abbreviations are the same as those defined in the legend for Fig. 2.

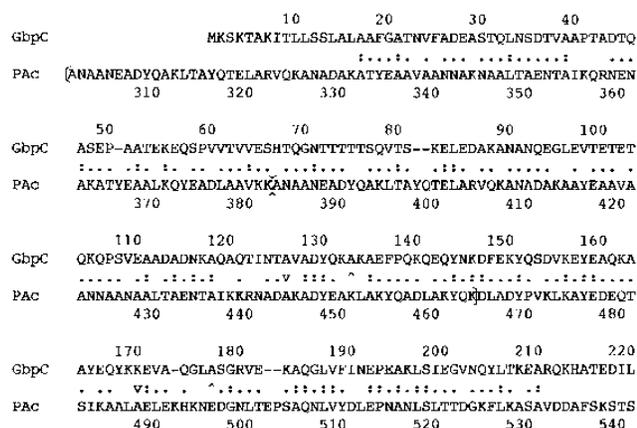


FIG. 4. Comparison of the GbpC protein and a portion of the PAC protein of *S. mutans*. Computer-generated alignment of the GbpC and PAC proteins allowing gaps (hyphens) is shown. Identical and similar amino acid residues are marked respectively as colons and dots between the two sequences. PAC is a protein of 1,565 amino acid residues with a cell-associated consensus structure at its carboxy terminus. The numbering of the sequences indicate the actual positions of each amino acid. Aligned with the sequence of the PAC protein is a portion of the surface-exposed domain, where three alanine-rich tandem repeats are present. Brackets on the PAC sequence denote the second and third alanine-rich repeats. v...v, similar region initially detected by the FASTA search.

mature protein with glucan-binding activity, we carried out Western blot analysis with antiserum to the 59-kDa GBP (27), whose antigenicity has been reported to be different from that for the 74-kDa Gbp (2). However, positive bands were detected in the extracts from both the wild type and the mutant of *S. mutans* in which the *gbpC* gene was deleted from its

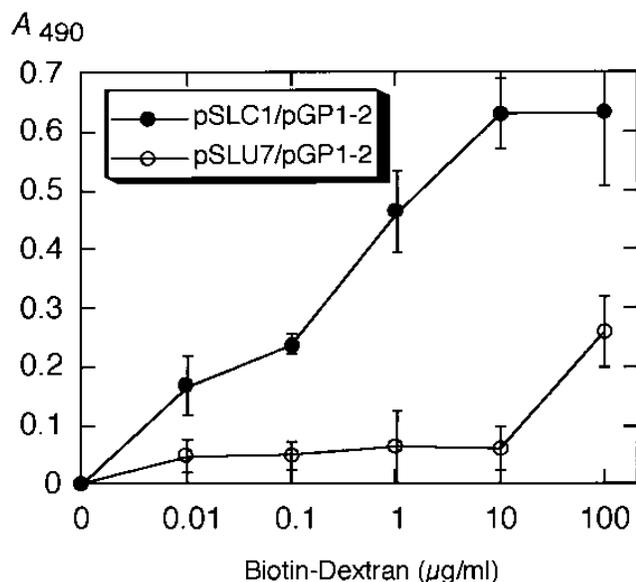


FIG. 5. Comparison of biotin-dextran binding by the GbpC clone and control cell extracts. Wells of ELISA plates were coated with equivalent amounts of protein for each extract and incubated with the indicated concentrations of biotin-dextran, and glucan binding was detected as described in Materials and Methods. The data presented are averages and standard errors of three independent determinations for each sample. Low-molecular-weight glucan (dextran T10) competitively inhibited biotin-dextran binding of the extract from the GbpC clone, and biotin alone did not promote color development (data not shown). Closed circles, extract from *E. coli* JM109 harboring pGP1-2 and pSLC1 (*gbpC* clone); open circles, extract from *E. coli* JM109 harboring pGP1-2 and pSLU7 (control); bars, standard errors.

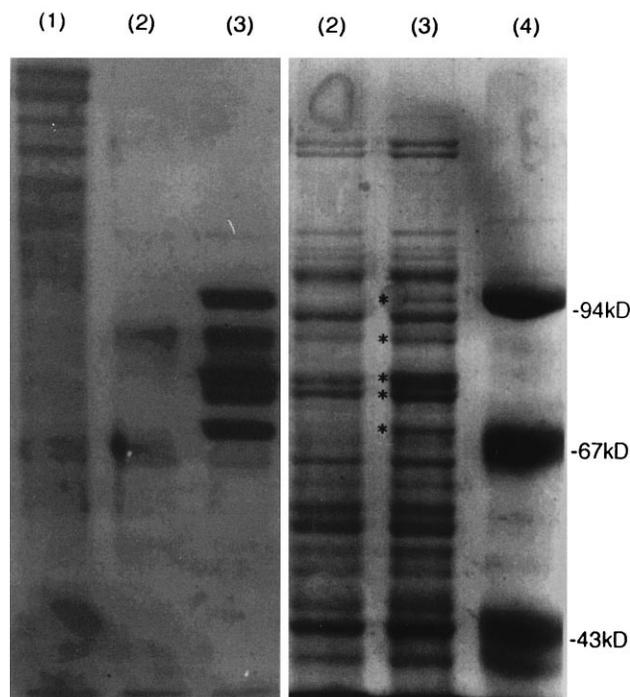


FIG. 6. The result of the Western blot analysis with anti-PAC serum (left) and the Coomassie brilliant blue-stained polyacrylamide gel (right). Cell extracts were prepared as described in Materials and Methods. Equivalent amounts of protein for each extract were applied in the wells. Lane 1, *S. mutans* whole-cell extract; lanes 2, *E. coli* JM109 harboring pGP1-2 and pSLU7 (control); lanes 3, *E. coli* JM109 harboring pGP1-2 and pSLC1 (*gbpC* clone); lane 4, a molecular weight standard. Asterisks denote bands corresponding to the positive bands in the Western blot.

chromosome and were not detected in the extract from the *E. coli gbpC* clone (data not shown). These results suggest that the protein encoded by the *gbpC* gene is distinct from the 59-kDa GBP (27) and that the GbpC protein is a novel GBP.

DISCUSSION

The dextran-dependent aggregation of 109cS was apparently different from the aggregation by insoluble glucans (33) or by 74-kDa Gbp (5) and is presumed to be independent of the GTFs or GBPs. However, in order to genetically confirm this, we constructed mutants by using strain 109cS in which previously cloned genes specifying GTFs and other proteins were inactivated on their chromosome. These constructs are *gtfB-gtfC-gtfD*, *gtfB-gtfC-gtfD-gbp*, *wapA* (7, 19), and *pac* (17) mutants, and they all exhibited rapid dextran-dependent aggregation (10). Therefore, we anticipated that a previously undescribed gene would be involved in the aggregation and initiated the random mutagenesis strategy.

Mutant 32A02 (Fig. 2) was not a simple pVA891 insertion mutant. The applied mutagenesis method employed entailed the random insertion of pVA891 into the host chromosome by a Campbell-type recombination event mediated between a *Sau3AI*-digested host chromosomal DNA fragment inserted in pVA891 and its chromosomal copy. However, when analyzing the chromosomes of mutants obtained in this experiment as well as those obtained with our previous two other mutagenesis approaches (24, 32), we found relatively large numbers of mutants conferred the target phenotype chromosomal rearrangements of which could not be explained by a single Camp-

bell-type insertion event. Mutant 32A02 (Fig. 2) was one such case, and more than 20 kb (maybe up to 50 kb) of the chromosomal region was deleted from its chromosome following mutagenesis. The constructed partial deletion mutants (Fig. 2), however, revealed that the region responsible for the aggregation was surprisingly small (only 2.2 kb) and is not able to code for a protein with a molecular mass of more than 80 kDa.

The *gbc* gene coding for the 59-kDa protein possessing glucan-binding activity was identified in this region. Previously, two GBPs in addition to GTFs have been purified from *S. mutans*. The first is a 74-kDa protein coded by the *gbc* gene (2) which has no sequence similarity to the *gbc* gene. The other is a 59-kDa GBP recently reported by Smith et al. (27), who described its purification and distinct antigenicity to the 74-kDa protein. The results of Western blot analysis revealed that the 59-kDa protein reported by Smith et al. was distinct from our GbpC protein. Therefore, our cloned *gbc* gene codes for a novel, third GBP, which is very likely a surface protein involved in the glucan-dependent aggregation.

Ma et al. (13) recently reported a 58- to 60-kDa GBL from *S. sobrinus* and suggested that a GBL is defined as a GBP capable of conferring upon a bacterium the ability to be aggregated by α -1,6 glucan. According to this definition, the GbpC protein can be classified as a GBL. Although no information concerning the amino acid sequence of the *S. sobrinus* GBL is available, its molecular weight and amino acid content of proline and alanine are similar to those of the *S. mutans* GbpC protein. Inhibitory effects of chelating agents or dextran T10 on the glucan-dependent aggregation of both organisms were also similar. Taken together with the above information, the GbpC protein may be a counterpart of the *S. sobrinus* GBL.

A FASTA homology search indicated similarity between the GbpC protein and a family of streptococcal surface proteins, and antiserum for PAc cross-reacted with the GbpC protein overexpressed in *E. coli*. Previous reports have stated that the SpaA protein from *S. sobrinus* is a GBP (1, 3, 6). Some common structures which are able to bind glucan may exist on these two proteins because of sequence similarity and antibody cross-reactivity, although the GbpC protein is obviously involved in the dextran-dependent aggregation but the PAc protein is not (see above). The positive bands in Western blots were unexpectedly multiple, and the same-sized protein bands could also be recognized in a Coomassie-stained gel. Even for the smallest band, its size was larger than the calculated molecular weight of the GbpC protein. How these multiple protein products were generated is not known at present. The *gbc* gene product may be toxic for *E. coli*, since the gene could not be subcloned into high-copy-number plasmids in the same direction as the *lac* promoter. Therefore, *E. coli* cells may have posttranscriptionally modified the GbpC protein in this case in order to detoxify it.

We expected the mutant LSL91 to exhibit the constitutive dextran-dependent aggregation because the *gbc* gene in this mutant could be transcribed from the *Em^r* gene promoter. However, like the parental strain, the mutant cells aggregated with dextran only after growth under stress conditions. These results suggest that expression of the *gbc* gene is essential but probably not sufficient for the dextran-dependent aggregation. The GbpC protein may need to be posttranscriptionally modified or requires other factors for aggregation. We have obtained some data to support this hypothesis. (i) Besides the mutant 32A02, we obtained about 40 of the *ddag⁻* mutants, which have not been characterized in detail. Some of them appeared to retain the intact *gbc* gene, since chromosomal DNA fragments analyzed by Southern hybridization following

digestions with several restriction enzymes were the same sizes as those of parental strain 109cS. (ii) We found that two serotype f strains, OMZ175 and 130f, did not aggregate with dextran T2000. These strains also carried the *gbc* gene as shown by Southern hybridization analysis. (iii) We have detected a small ORF downstream from the *scrB* gene previously characterized in our labs (22, 23) with strain GS-5. When a mutant was constructed with the ORF inactivated in the chromosome of strain 109cS, this mutant constitutively exhibited the dextran-dependent aggregation under nonstress conditions. This ORF is an example of a possible factor involved in aggregation. However, we do not yet know whether this ORF controls transcription of the *gbc* gene. We have no data regarding transcriptional regulation for any of the mutants or the parental strain at present. Moreover, the other factors regulating the dextran-dependent aggregation remain to be determined. Such further studies are currently in progress.

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