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Genes responsible for dextran-dependent aggregation of *Streptococcus sobrinus* strain 6715

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Introduction: *Streptococcus* sobrinus exhibits more significant dextran-dependent aggregation mediated by glucan-binding proteins than *Streptococcus mutans*. We have identified four glucan-binding protein C gene (*gbpC*) homologues designated as *gbpC1*, *gbpC2*, *dblA* and *dblB* in *S. sobrinus* in contrast to the single gene *gbpC* in *S. mutans.* We attempted to determine which gene is most responsible for the dextran-dependent aggregation of *S. sobrinus.*

Methods: We introduced mutation with a chemical mutagen, 1-methyl-3-nitro-1-nitrosoguanidine, into *S. sobrinus* strain 6715 and analysed the four *gbpC* homologous gene sequences in the parental strain 6715 and an obtained aggregation-negative mutant NUM-Ssg99. We also examined the localization of proteins encoded by these genes in the mutant NUM-Ssg99.

Results: The nucleotide sequences of the *gbpC1*, *gbpC2* and *dblA* genes in NUM-Ssg99 were 100% identical to the homologous genes in parental strain 6715. In contrast, a truncated mutation was detected in the *dblB* gene and the mutant protein devoid of the LPXTG motif was confirmed by Western blot analysis to be released into the extracellular milieu.

Conclusion: We conclude that the dblB gene among the four GbpC homologous protein genes is most responsible for aggregation in strain 6715.

Key words: chemical mutagenesis; dextran-dependent aggregation; gbpC/dbl genes; glucan-binding proteins; IS *1548*-like sequence; *Streptococcus sobrinus*

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Streptococcus mutans and *Streptococcus sobrinus* are regarded as the principal etiological agents of human dental caries. These two organisms express common putative virulence factors involved in dental caries, e.g. glucosyltransferases, surface protein antigens, which were investigated at a molecular level in recent years. Although *S. sobrinus* is carried by a minority of the population, the cariogenic potential in rats of *S. sobrinus* was reported to be greater than that of *S. mutans* (5). However, molecular analysis of virulence factors is less advanced in *S. sobrinus*. For example, glucan-binding proteins (2) A, B and D encoded by the *gbpA* (1), *gbpB* (4, 17) and *gbpD* (25) genes, respectively, have been identified in *S. mutans* but their homologues in *S. sobrinus* have not yet been identified. Glucan-binding protein C encoded by the *gbpC* gene was initially identified as the protein solely involved in dextran (α-1,6 glucan)-dependent aggregation of *S. mutans* (23). In contrast, the *S. sobrinus* genes involved in this phenomenon have been identified only recently (15) even though *S. sobrinus* has been known to exhibit more active dextran-dependent aggregation than *S. mutans* (6).

Two essential traits for the proteins responsible for the dextran-dependent aggregation phenotype are dextran-binding activities and localization on the cell surface. Very recently, we identified four *gbpC* homologues designated as *gbpC1*, *gbpC2*, *dblA* and *dblB* in *S. sobrinus* (21). The GbpC1 protein was not considered a candidate for this property because, even though it bound to Sephadex (cross-linked

dextran), it exhibited no dextran-binding activity in conventional assays (15). According to sequence analyses of these four genes, all of these homologues encoded cell wall-anchored surface proteins possessing LPXTG motifs by which sortase mediates tethering of the proteins to cell wall peptidoglycans. Therefore, we previously suggested that the GbpC2, DblA and DblB protein homologues were logical candidates involved in dextran-dependent aggregation of this organism (21). The best way to resolve the question as to which of the three candidates most significantly contributes to dextran-dependent aggregation would be to introduce gene-directed mutations into strains of *S. sobrinus*. However, it is difficult to construct knockout mutants in *S. sobrinus* by introducing a specifically inactivated gene fragment, e.g. an allelic exchange following transformation of the DNA fragment. This is also the reason why molecular analyses in *S. sobrinus* have been problematic as described above.

Therefore, we attempted to introduce dextran-induced aggregation mutations into *S. sobrinus* 6715 with a chemical mutagen, 1-methyl-3-nitro-1-nitrosoguanidine, and analysed the resulting four *gbpC* homologous gene sequences. We also examined localization of the protein products of these genes relative to tethering to the cells or release into the extracellular milieu in the mutants. In this manner, we discuss the relative contributions of these genes to the dextran-induced aggregation phenotype.

Materials and methods

Bacterial strains and plasmids

S. sobrinus strain 6715 (serotype *g*) and its mutant NUM-Ssg99, strains K1R (a derivative of 6715), B-13N and OMZ176 were used for this study. Strain NUM-Ssg99 had been previously isolated as a 1-methyl-3-nitro-1-nitrosoguanidine-induced mutant deficient in dextran-dependent aggregation by M. Hirasawa (unpublished data). The streptococci were maintained on Todd–Hewitt (TH) broth/agar plates and cultured in brain–heart infusion (BHI) broth or BTR broth (1% tryptone peptone, 0.1% bacto yeast extract, 0.05% sodium thioglycollate, 0.61% K_2HPO_4, 0.2% KH_2PO_4, 1 mM $MgSO_4$, 0.1% $MnSO_4$, 0.2% carbon source) (24). *Escherichia coli* strain TOP10 obtained from a commercial supplier (Invitrogen, Carlsbad, CA), was used as a host for plasmid pBAD/His and its derivatives.

Mutagenesis with 1-methyl-3-nitro-1-nitrosoguanidine

S. sobrinus 6715, which was resistant to streptomycin, was cultured in BHI broth at 37°C in a candle jar for 24 h. The growing cells were harvested, washed with 0.05 M Tris–HCl buffer (pH 7.2), suspended in the fresh BHI broth containing 1-methyl-3-nitro-1-nitrosoguanidine (final 0.15 mg/ml), and the mixture was incubated at 37°C in a candle jar for 2 h. After mutagen exposure, culture samples were diluted and spread on a Mitis Salivarius agar plate. All isolates were confirmed to be resistant to streptomycin.

Polymerase chain reaction amplification, nucleotide sequencing and sequence analysis

The regions corresponding to the glucan-binding protein genes *gbpC1*, *gbpC2*, *dblA* and *dblB*, in strains NUM-Ssg99, K1R, B-13N and OMZ176 were amplified and sequenced with primers used to identify these genes in strains 100-4 and 6715 (15, 21). Primers to amplify and sequence the sortase gene *srtA* in *S. sobrinus* were designed based on the *S. mutans srtA* gene sequence, and the *S. sobrinus srtA* gene sequences were determined in strains 6715, NUM-Ssg99, K1R and OMZ176. The nucleotide sequence region deleted in the chromosome of strain NUM-Ssg99 was obtained from strain 6715 as several amplicons encompassing the entire deleted region by the polymerase chain reaction-based genome walking method with the Universal GenomeWalker kit (BD Biosciences Clontech, Palo Alto, CA) as described previously (19). Sequence analyses were carried out with DNASIS-MAC (Hitachi Software Engineering, Yokohama, Japan) and GENETYX-MAC (Genetyx Corporation, Tokyo, Japan) programs.

Protein sample preparation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis

Preparation of wall protein extracts from *S. sobrinus* strains was carried out using a peptidoglycan *N*-acetylmuramoyl hydrolysing enzyme as described previously (22) because the GbpC1, GbpC2, DblA and DblB proteins are covalently linked to the peptidoglycan layer of the *S. sobrinus* cell wall. The procedure was a slightly modified protocol originally reported by Homonylo-Mcgavin and Lee (11, 12). Briefly, the *S. sobrinus* cells were grown with BTR-Glucose (24) broth and harvested. The washed cells were disrupted by ultrasonication and subjected to low-speed centrifugation (3000 ***g***, 2.5 min) with a microfuge (M150; Sakuma, Tokyo, Japan) to remove undisrupted cells. The supernatant fluid contained disrupted wall particles. To release wall proteins, washed wall particles were digested with *N*-acetylmuramidase SG™ (2000 U/mg protein; Seikagaku Corporation, Tokyo, Japan) at 50°C and pH 6.5, and subjected to high-speed centrifugation (15,000 ***g***, 5 min). The resulting supernatants were obtained as the wall sample preparations. Protein assays were carried out using the Lowry method. Wall protein concentrations in the preparations were determined by subtracting the amounts of the added *N*-acetyl muramidase SG™ protein in a control tube lacking cell wall particles.

A method to prepare concentrated *S. sobrinus* glucan-binding proteins from culture supernatants was simplified from that reported by Smith et al. (27) as described previously (15). The crude extracts were prepared from the induced *E. coli* clones previously designated (21) as ZBM1, ZD67, ZCQ1 and ZD54, respectively, expressing the extracellular domains of the GbpC1, GbpC2, DblA and DblB proteins. The GbpC2 and DblB proteins were purified with Ni-Sepharose 6 Fast Flow resin (GE Healthcare Biosciences KK, Tokyo, Japan) as described previously, and the purified proteins were used to immunize female Japanese white rabbits to prepare anti-ZD67 (GbpC2) and anti-ZD54 (DblB) sera (Operon Biotechnologies, Inc., Tokyo, Japan).

These streptococcal sample extracts and the crude extracts from the induced *E. coli* cells were mixed with sodium dodecyl sulfate sample buffer and frozen for subsequent electrophoretic analysis using the Laemmli gel system with acrylamide gels.

Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the separated proteins were electrophoretically transferred and Western blot analyses with anti-sera described above, anti-ZCQ1 (DblA), anti-GBP-2, anti-GBP-3 or anti-GBP-5, were carried out as described previously (19). The last three anti-sera (27) were kindly provided by D. J. Smith.

Dextran-dependent aggregation

BHI or BTR broths were used to cultivate *S. sobrinus* 6715, NUM-Ssg99 and B-13N cells and dextran-dependent aggregation of the 15-h cultures was observed visually as described previously (19). The cultures were divided into two 0.5-ml portions and

dextran T2000 was added (100 μg/ml final concentration) to one of them. Each pair of tubes was photographed following swirling for several seconds.

Results

Dextran-dependent aggregation-negative mutant induced by a chemical mutagen

Gene-specific mutagenesis mediated by a Campbell-like integration of plasmid or allelic exchange with a linear DNA fragment is difficult in *S. sobrinus*, although an example was reported (3). Therefore, a chemical mutagen, 1-methyl-3-nitro-1-nitrosoguanidine, was applied for mutagenesis of *S. sobrinus* as reported previously using ethyl methanesulfonate (26). A mutant, designated as NUM-Ssg99, did not aggregate when the glucose-grown cells were exposed to dextran T2000 or when grown with sucrose under the condition described by Gibbons and Fitzgerald (8). This aggregation-defective mutant NUM-Ssg99 exhibited glucosyltransferase and plaque-forming activities. These activities were similar to those of the parental strain 6715, indicating that the genes encoding soluble and insoluble glucan-producing enzymes were expected to be intact. As suggested by our previous studies (15, 21) indicating that one of the four *gbpC* homologues was likely involved in dextran-dependent aggregation of *S. sobrinus*, we analysed whether or not a mutation was generated within any of these *gbpC* homologues in MUM-Ssg99.

Sequence analyses of the four *gbpC* homologues in strain 6715 and mutant NUM-Ssg99

The *gbpC1* and *gbpC2* genes are tandemly located on the chromosome of strain 6715, and the *dblA* gene was also located immediately upstream of the *dblB* gene (21). The nucleotide sequence regions containing the *gbpC1*, *gbpC2* and *dblA* genes in NUM-Ssg99 were 100% identical to those corresponding to parental strain 6715. In contrast, a mutation was detected in the *dblB* gene of NUM-Ssg99. This mutation appeared not to result from a simple nucleotide substitution or a single nucleotide deletion/insertion when comparing both sequences downstream from nucleotide position 4002 (Fig. 1). An amino acid sequence deduced from the 432-base-pair (bp) sequence downstream from nucleotide position 4002 in NUM-Ssg99 was not similar to any regions of the four GbpC homologous protein sequences. The results of a homology search against the EMBL-GenBank-DDBJ databases suggested that the amino acid sequence might be the C-terminal region of the histidinol-phosphate aminotransferase protein encoded by the *hisC* gene homologue in *S. sobrinus*. To identify the chromosomal locations of the *dblB* and putative *hisC* genes, Southern hybridization analyses following pulse field gel electrophoresis with restriction enzymes *Apa*I-, *Sma*I- and *Xho*I-digested chromosomal DNA samples from both strain 6715 and mutant NUM-Ssg99 were carried out (data not shown). A positive band when probed with *dblB* was unique and was the same size as that when probed with *hisC* 3′ fragment in each digested sample from 6715, suggesting that both the *dblB* and *hisC* genes reside on a single fragment digested with any of the three restriction enzymes. When analysed with NUM-Ssg99 chromosomal DNA the results were the same. However, the sizes of the positive bands in the NUM-Ssg99 chromosomal DNA samples digested with any of the three restriction enzymes were approximately 10 kilobases (kb) smaller than those in the 6715 samples, suggesting that a relatively wide range of a DNA region was deleted in the chromosome of mutant NUM-Ssg99 following mutagenesis with the chemical mutagen 1-methyl-3-nitro-1-nitrosoguanidine. Since a deleted region was suggested in the mutant, we performed polymerase chain reaction-based genome walking downstream from the 3′ region of the *dblB* gene and upstream from the 3′ region of the putative *hisC* gene with the parental strain 6715 sequencing the amplified fragments step by step. We then determined the 10,326-bp deleted nucleotide sequence, which encodes five putative genes including two putative cation-transporting P-type ATPase genes, two hypothetical genes, and the 5′ region of the putative *hisC* gene.

The 10,326-bp deletion resulted in a truncation of the *dblB* gene and the generation of a *dblB::hisC* fusion gene in mutant NUM-Ssg99. Therefore, the DblB::HisC fusion protein devoid of the LPXTG motif was expected to be released into the extracellular milieu without attachment to the cell wall peptidoglycan layer. This may be the reason why this mutant does not exhibit dextran-dependent aggregation.

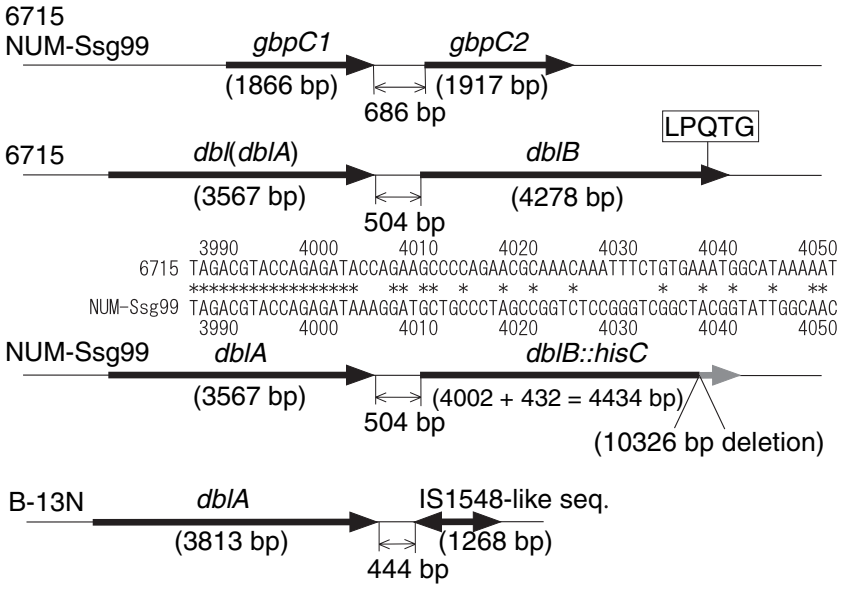

Fig. 1. Mutation of the *dblB* gene in NUM-Ssg99. Nucleotide sequence of the *dblB* gene in NUM-Ssg99 downstream from the nucleotide position 4002 was distinct from that in parental strain 6715. The *dblB::hisC* fusion gene resulted from a 10,326-bp deletion with the LPQTG-motif coding region. The *dblB* gene in strain B-13N was replaced with an *IS1548*-like sequence.

Comparison of aggregation phenotype between strain 6715 and mutant NUM-Ssg99

Mutant NUM-Ssg99 was isolated with a dextran-dependent aggregation-negative phenotype and did not exhibit significant clumping following the addition of dextran T2000 into a BHI culture. The appearance of NUM-Ssg99 was compared with the similarly treated parental strain 6715 (Fig. 2). We never use TH broth to observe

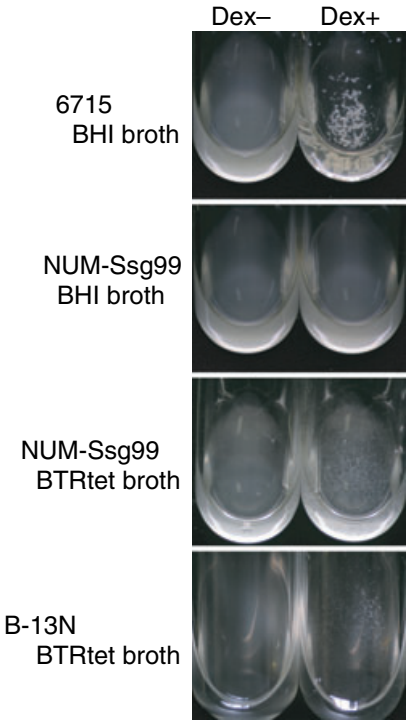

Fig. 2. Dextran-dependent aggregation of *Streptococcus sobrinus* strain 6715, its mutant NUM-Ssg99 and strain B-13N in the presence of exogenous Dextran T2000.

dextran-dependent aggregation because cells in TH cultures exhibit aggregation in the absence of exogenous dextran T2000. This results from insoluble glucan formation mediated by *S. sobrinus* glucosyltransferases using the contaminated sucrose present in TH broth as a substrate. Although NUM-Ssg99 harbors a mutation in the *dblB* gene as described above, the other *gbpC* homologues appear to be intact. Therefore, we attempted to detect cell clumping in BTR-tet (tetracycline 0.19 μg/ml final concentration) cultures in which we could previously detect dextran-dependent aggregation in the normally poorly aggregating *S. mutans* (23). NUM-Ssg99 exhibited subtle but consistent cell clumping under these growth conditions in the presence of dextran T2000 (Fig. 2).

Sequence analyses of the *gbpC* homologues in strains K1R, OMZ176 and B-13N

Two other dextran-dependent aggregation-negative strains of *S. sobrinus*, K1R and OMZ176, were included in our frozen stock culture collection. Therefore, we analysed the sequences of the *gbpC* gene homologues in these strains as well as the *srtA* genes encoding sortase enzymes mediating the tethering of LPXTG motif proteins to cell wall peptidoglycan. The intactness of the genes, accession numbers and aggregation phenotypes in these strains as well as in strains 6715 and NUM-Ssg99 are summarized in Table 1. The four *gbpC* homologous genes in strain K1R were all intact but a frameshift mutation was detected in the *srtA* gene. In contrast, the *srtA* and *gbpC1* genes in strain OMZ176 were intact but the *gbpC2*, *dblA* and *dblB* genes were mutated with a single adenine nucleotide insertion, a single adenine nucleotide insertion, and 736 nucleotide deletions, respectively. These two strains did not exhibit dextran-dependent aggregation even under conditions in which NUM-Ssg99 exhibited aggregation as described above. These results suggest that (i) the *gbpC1* gene is not sufficient to account for dextran-dependent aggregation, (ii) the other three homologous genes were possibly involved, and (iii) the *srtA* gene was essential for dextran-dependent aggregation.

S. sobrinus strain B-13N was previously isolated by K. Takada as a B-13 derivative producing high levels of extracellular water-insoluble glucan (7) (The strain was abbreviated as *S. mutans* strain B-13 in that paper). Recently, we found that this strain did not exhibit dextran-dependent aggregation following the addition of dextran T2000 into a BHI culture but we detected cell clumping in BTR-tet cultures similar to mutant NUM-Ssg99 (Fig. 2). We determined the sequence downstream from the *dblA* gene in this strain. However, the *dblB* gene sequence was not detected in this region, instead a sequence similar to *IS1548* (9) containing an open reading frame encoding a transposase-like amino acid sequence was identified. *IS1548* inserted within the hyaluronidase gene *hylB* was initially identified in some strains of group B streptococci and the *IS1548*-like sequences were also detected in group A streptococci and pneumococci (9). The sequence downstream from the *IS1548*-like sequence in strain B-13N was highly similar to that approximately 4 kb downstream from the 3′ end of the *dblB* gene in strain 6715. This suggested that the *IS1548*-like sequence appeared to be inserted into the region upstream from the *dblB* gene and subsequently the *dblB* gene plus a downstream 4-kb region may be deleted in strain B-13N. Southern hybridization analysis confirmed that the *dblB* gene was absent in the chromosome of strain B-13N and that the *IS1548*-like sequence was not present in strain 6715 and the mutant NUM-Ssg99.

Expression of GbpC homologous proteins in 6715 and NUM-Ssg99

Since GbpC2, DblA and DblB proteins were expected to be involved in dextran-dependent aggregation, we attempted to determine whether these proteins of 6715 and NUM-Ssg99 were tethered to the cell wall or released into the culture supernatants by Western blot analyses using anti-ZD67 (GbpC2), anti-ZCQ1 (DblA) and anti-ZD54 (DblB) sera. Wall proteins and supernatant Sephadex-binding proteins of 6715 and NUM-Ssg99 were prepared as described in the Materials and methods section. Western blot analysis with anti-GbpC2 serum detected no protein bands in the wall and supernatant samples from both strains 6715 and NUM-Ssg99 (data not shown), suggesting that GbpC2 protein was not expressed or was expressed at very low levels under these growth conditions. The analysis with anti-DblA serum detected clear positive bands around 150 kDa, in wall samples from both 6715 and NUM-Ssg99 with almost the same intensities (left panel of Fig. 3). These 130- and 100-kDa bands probably represented partially degraded proteins. The 150-kDa positive bands were also detected in the supernatant samples from 6715 and NUM-Ssg99, although the intensity of the band in NUM-Ssg99 was

Table 1. Summary of accession numbers, intactness of the genes, and aggregation phenotypes (ddag) in several *Streptococcus sobrinus* strains

Strain	*gbpC1*	*gbpC2*	*gbpA*	*dblB*	*srtA*	ddag
6715	AB294108	AB294108	AB294109	AB294109	AB281282	+++
K1R	AB281279	AB453912	AB281281	AB453913	AB281283	–
NUM-Ssg99	AB453914	AB453915	AB302322	AB302322	AB453916	– (+)
OMZ176	AB241126[1]	AB368854	AB237535[1]	AB453917	AB105865	–
B-13N	nd	nd	AB465738	AB465738	nd	– (+)
100-4	AB237533[1]	nd	AB237534	AB237534	nd	+++

Underlined accession numbers represent the gene with mutation. The other accession numbers contain the intact genes.
[1]From Ref. 15, the other accession numbers were registered or updated with this study.
nd, Not determined.

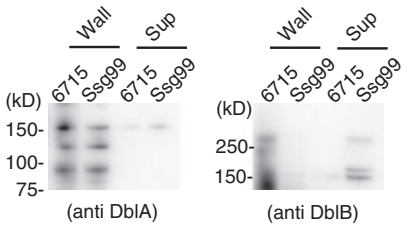

Fig. 3. Western blot analyses of DblA and DblB proteins in wall and supernatant fractions isolated from *Streptococcus sobrinus* strain 6715 and its mutant NUM-Ssg99. Samples were prepared as described in the text. The apparent size, by migration, of proteins DblA and DblB was larger than their calculated molecular sizes as described previously (15). Amounts of wall protein samples applied per lane were 410 ng for anti-DblA and 120 ng for anti-DblB analyses. Volumes of supernatant samples per lane were 2 μl for anti-DblA and 4 μl for anti-DblB analyses.

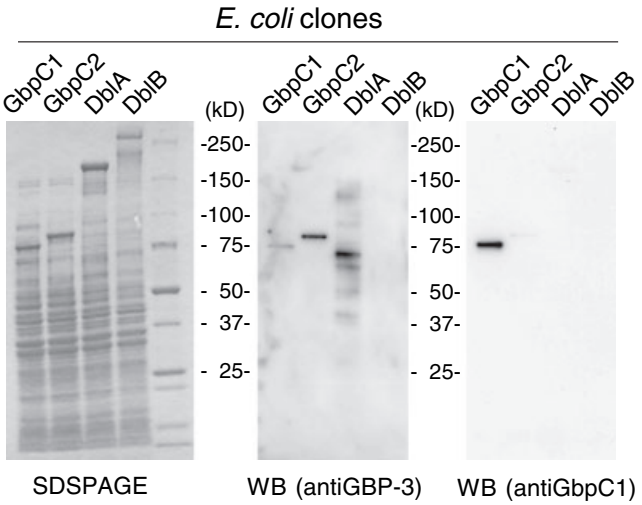

Fig. 4. Western blot analysis of *Escherichia coli gbpC1*, *gbpC2*, *dblA* and *dblB* clones with anti-GBP-3 and anti-GbpC1 sera. Each *E. coli* clone apparently overexpresses proteins encoded by the transformed gene, as indicated with extra protein bands in each lane of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis to which 2 μl crude extract of each sample was applied. Crude extracts (300 μl) were prepared from 1.5-ml cultures of ZBM1, ZD67, ZCQ1 and ZD54, respectively, expressing the extracellular domains of the GbpC1, GbpC2, DblA and DblB proteins with the calculated molecular sizes of 65, 67, 138 and 150 kDa, respectively. Major protein bands derived from each cloned DNA fragment apparently migrated larger than their calculated molecular sizes, especially for the DblB protein. For Western blot analysis, 2 μl of 1/100 diluted extract was used.

slightly higher than that in 6715. In contrast, the analysis with anti-DblB serum revealed positive bands in a wall sample of 6715 and in a supernatant sample of NUM-Ssg99 (right panel of Fig. 3). These results, together with sequence analyses, suggested that the DblB protein tethered to the cell wall was responsible for significant dextran-dependent aggregation of 6715, and that the DblA protein may contribute to this phenomenon to a minor extent.

Relationships between the four GbpC homologuos proteins (GbpC1, GbpC2, DblA and DblB) and previously reported glucan-binding proteins (GBP-2, GBP-3 and GBP-5)

Smith et al. (27) previously reported three glucan-binding proteins purified from *S. sobrinus* 6715 culture supernatants and designated them GBP-2, GBP-3 and GBP-5. They kindly provided us with antisera against these proteins and unpublished information concerning the partial N-terminal sequences of the proteins GBP-3 and GBP-5. The former sequence was found in GbpC1 as a 100% identical 13 amino acid residues, which corresponds to the N-terminal sequence of the putative mature GbpC1 protein following a signal peptide sequence predicted by the SIGNALP 3.0 server (available on an internet site: http://www.cbs.dtu.dk/services/SignalP/), and was not found in the other GbpC homologues. We analysed crude extracts of *E. coli* clones expressing each GbpC homologous protein with Western blots using the three antisera. GBP-2 and GBP-5 antisera did not cross-react with any of the *E. coli* clones. GBP-3 antiserum cross-reacted with not only ZBM1 (GbpC1) but also with the ZD67 (GbpC2) and ZCQ1 (DblA) clones (Fig. 4). These results suggest that the GBP-3 protein is very likely a released form of the GbpC1 protein.

Discussion

The nucleotide sequence analyses of four *gbpC* homologous genes in mutant NUM-Ssg99 mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine clearly indicated that the gene responsible for dextran-dependent aggregation typically observed among strains of *S. sobrinus* species was the *dblB* gene. In addition, strain B-13N exhibited the same phenotype as the mutant NUM-Ssg99. In contrast, the *gbpC2* and *dblA* genes of the mutant were intact. Therefore, we considered that this mutant may exhibit dextran-dependent aggregation depending on its growth condition, e.g. cells grown in BTR-tet medium, in which we previously observed dextran-dependent aggregation of *S. mutans* (23). This mutant exhibited subtle but consistent cell clumping under this growth condition (Fig. 2). Therefore, the GbpC2 and DblA proteins are possibly involved in this aggregation phenotype in strain 6715. However, we could not detect GbpC2 expression in the cell walls of strain 6715 by Western blot analysis with anti-GbpC2 serum. Collectively from these findings, we conclude that loss of the DblB protein from the wall of NUM-Ssg99 is the reason for the attenuation of its consistent aggregation phenotype. We can also propose a contribution of the DblA protein to dextran-dependent aggregation in *S. sobrinus* species as suggested in our recent report (15) with strain 100-4. When we compared relative expression of DblA proteins in wall samples with Western blot analysis between strains 100-4 and 6715, the expression level in strain 100-4 was approximately fivefold higher than in strain 6715. We previously observed relative to *S. mutans* GbpC expression (22) that the mutant LSDVR1, which exhibits a constitutive dextran-dependent aggregation phenotype comparable with *S. sobrinus*, expressed 5- to 10-fold greater GbpC protein than the parental strain. Therefore, these levels of overexpression of wall-anchored glucan-binding proteins may significantly contribute to the apparent constitutive dextran-dependent aggregation phenotype of these streptococcal cells. In addition, we recently found that some *S. sobrinus* strains do not harbor the *dblB* gene (unpublished results from the 86th General Session of the International Association for Dental Research, abstract no. 719). These strains exhibited dextran-dependent aggregation similar to those of strains 100-4 and 6715. The identity of the genes which are responsible in these strains is currently under investigation.

Therefore, we also suggest that the role of the *dblA* gene and/or other genes in this phenotype may be dependent on strain differences in *S. sobrinus.*

Mutagenesis with a chemical reagent like 1-methyl-3-nitro-1-nitrosoguanidine generally induces nucleotide substitutions. However, our results demonstrated neither nucleotide substitution mutations nor complex chromosomal rearrangements but instead mutations with a relatively wide range of nucleotide deletions (more than 10 kb). Although several genes reside in this region, we could not detect any phenotypic changes and do not know how this deletion occurred. The *dblB* gene in strain B-13N appeared to be replaced with a *IS1548*-like sequence, which was not present in the chromosome of the mutant NUM-Ssg99. Other *IS*-sequences or transposons might be involved following a nucleotide substitution in a non-essential gene of the mutant NUM-Ssg99. As a result, this might induce activation of a transposase as reported in *S. mutans* (16, 20), although these mobile genetic elements have not been reported to actively transpose in the *S. sobrinus* chromosome. Strain B-13 was reported to exhibit typical dextran-dependent aggregation (28). Therefore, during laboratory transfer, an *IS1548*-like sequence may have been spontaneously activated and transposed within strain B-13.

In contrast to the single gene *gbpC* in *S. mutans*, four *gbpC* gene homologues, *gbpC1*, *gbpC2*, *dblA* and *dblB* are present in *S. sobrinus*. This is similar to the case in glucosyltransferase genes encoding enzymes that mediate the synthesis of water-soluble glucans, i.e. the sole gene *gtfD* in *S. mutans* (13) vs. three *gtfD* homologous genes *gtfS*, *gtfT* and *gtfU* in *S. sobrinus* (10). In *S. mutans*, expression of the *gbpC* and *gtfD* genes is coordinately regulated by the same factors (14). It will be of interest to determine how the *gbpC* and *gtfD* homologous genes in *S. sobrinus* are regulated. The four *gbpC* gene homologues appear to be paralogues in this instance. We could not, however, determine whether these four *gbpC* gene homologues were paralogues or were orthologous to those in the other species (e.g. *Streptococcus criceti*), because sequence analysis of these genes in a species similar to *S. sobrinus* has not yet been carried out. Sequence analysis of these homologues in *S. criceti* is currently in progress in our laboratory.

Glucan-binding proteins that could mediate dextran-dependent aggregation in *S. sobrinus* were reported in the late 1970s (18) and the observation that *S. sobrinus* exhibits more active dextran-dependent aggregation than *S. mutans* was subsequently confirmed (6). However, the candidate genes responsible for this phenotype in *S. sobrinus* were not identified until recently (15, 21) and the genes corresponding to the previously reported glucan-binding proteins (2, 27) of *S. sobrinus* have not been characterized. We suggested that GBP-3 was most probably a product of the *gbpC1* gene, and may also be related to the *gbpC2* and/or *dblA* genes (Fig. 4). The GBP-2 and GBP-5 proteins were not likely encoded by any of these four *gbpC* gene homologues and which genes encode these proteins remains to be resolved. Since strain K1R, a sortase-negative derivative, and the *gbpC2-dblA-dblB*-negative strain OMZ176 exhibited no dextran-dependent aggregation, proteins involved in this phenotype should be LPXTG-motif proteins.

It is difficult to introduce a specifically inactivated gene fragment into *S. sobrinus*. Therefore, we performed the mutagenesis with the chemical mutagen. Although we cannot exclude a possibility for the presence of candidates other than these GbpC/Dbl proteins, we conclude that DblB protein is most responsible among the four GbpC homologous proteins for the aggregation of strain 6715, and further suggest that the *dblA* and/or *gbpC2* genes may contribute to this phenotype depending on strain differences in *S. sobrinus*.

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