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Original Article

gbpC Gene Repertoire Variation among Mutans Streptococci

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Abstract

The human dental caries pathogen *Streptococcus mutans* harbors one glucan-binding wall-anchored protein gene, *gbpC*, and another human pathogen, *Streptococcus sobrinus*, has 4 *gbpC* homologues. As no 4 *gbpC* homologues have so far been detected in other mutans streptococci, however, we cannot say whether those in *S. sobrinus* are paralogous or orthologous. Therefore, the purpose of this study was to identify and sequence the *gbpC/dbl* genes in *Streptococcus downei* and *Streptococcus criceti*. The findings revealed that the former harbored 5 *gbpC/dbl* homologues and the latter 4. In addition, another *dbl* homologue, the *dblC* gene, was found in some strains of *S. sobrinus*. We performed a phylogenetic analysis with the *gbpC/dbl* genes in these mutans streptococci. These results indicate that the *gbpC* and *dbl* genes have an orthologous relationship.

Key words: Mutans streptococci — *gbpC* — *dbl* — Glucan-binding wall-anchored proteins — Phylogenetic analysis

Introduction

Mutans streptococci (MS), major pathogens associated with dental caries not only in human, but also in monkey and rodent[7], commonly conserve genes encoding major virulence factors involved in dental caries, including surface protein antigens (SpaP, PAc, antigen I/II) and glucosyltransfereses[2]. In addition to these two factors, *Streptococcus mutans* and *Streptococcus sobrinus* express other virulence-related factors in human, including the glucan-binding cell wall-anchored proteins encoded by the glucan-binding protein C (*gbpC*) and dextran-binding lectin (*dbl*) genes[10,12]. The *gbpC* gene was initially identified as the gene solely involved in dextran (α-1,6 glucan)-dependent aggregation of

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S. mutans[12]. No homologous sequences were detected in the genome of this organism[1,8]. In contrast, 4 *gbpC* gene homologues (*gbpC1*, *gbpC2*, *dblA*, and *dblB*) were detected in *S. sobrinus*, which can exhibit more active dextran-dependent aggregation than *S. mutans*[4,11]. The monkey (Macaca fascicularis) and hamster microbial parasites *Streptococcus downei* and *Streptococcus criceti* are more closely related to *S. sobrinus* than *S. mutans* according to phylogenic analysis with 16S ribosomal RNA sequences and base composition of deoxyribonucleic acid[3,6]. Therefore, these two organisms are expected to harbor multiple *gbpC* gene homologues. The purpose of this study was to identify and sequence the *gbpC/dbl* genes in *Streptococcus downei* and *Streptococcus criceti*. Furthermore, we discuss the evolutionary development and significance of *gbpC* homologues in MS.

Materials and Methods

1. Bacterial strains

S. sobrinus strain OM55d (serotype *d* clinical isolate from our laboratories), *S. downei* strain Mfe28 (ATCC33748), and *S. criceti* HS6 (ATCC19642) were used in this study. The streptococci were maintained and cultured in Todd-Hewitt or Brain Heart Infusion broth/agar plates.

2. PCR amplification, nucleotide sequencing, and sequence analysis

Most of the regions corresponding to the *gbpC1/gbpC2* and *dblA/dblB* genes in *S. sobrinus* strain OM55d and *S. downei* strain Mfe28 were amplified with primers previously used to identify these genes in *S. sobrinus* strains[5]; some regions that could not be amplified with these primers were obtained by the PCR-based genome walking method (BD Biosciences Clontech, Palo Alto, CA) as described previously[9]. The *gbpC2* and *dblA* internal fragments of *S. criceti* HS6 were amplified with degenerate primers YF2 and YR2 as previously described[9] and the flanking regions were amplified by the genome walking method as described above. The amplified fragments were purified and directly sequenced with the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and the ABI PRISM Genetic Analyzer 3130 Avant (Applied Biosystems, Foster City, CA). The sequence analyses were carried out with the DNASIS-Mac (Hitachi Software Engineering, Yokohama, Japan), GENETYX-MAC (Genetyx Corp., Tokyo, Japan), and ClustalW (DDBJ) programs. A phylogenetic tree was drawn with the TreeExplorer program (Ver. 2.12) based on the ClustalW results.

3. Nucleotide sequence accession numbers

The nucleotide sequence data reported in this communication will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers AB237533 (100-4 *gbpC1/gbpC2*), AB237534 (100-4 *dblA/dblC*), AB576862 (OM55d *gbpC1/gbpC2*), AB447999 (OM55d *dblA/dblC*), AB557647 (MFe28 *gbpC1/gbpC3/gbpC2*), AB557646 (MFe28 *dblA/dblB*), AB237528 (HS6 *gbpC2*), and AB557645 (HS6 *dblA/dblB*)

Results

1. Amplification and nucleotide sequences of *gbpC/dbl* gene homologues in *S. sobrinus* strain OM55d

The *gbpC1*, *gbpC2*, and *dblA* genes of *S. sobrinus* strain OM55d were amplified and sequenced with the primers used to identify these genes in strains 100-4 and 6715[5,11]. In contrast, the *dblB* gene could not be amplified with these primers. Therefore, fragments downstream from the *dblA* gene were obtained by genome walking. A 4,026-bp open reading frame (ORF) sequence similar to those of the *dblA* or *dblB* genes but distinct from these two sequences was present 211 bp downstream from the *dblA* gene. This gene was 58.4% identical to the 6715 *dblA* gene over 3,621 bps and 58.5% identical to the 6715 *dblB* gene over 3,956 bps. Therefore, this gene was designated as the *dblC* gene. In addition, a highly

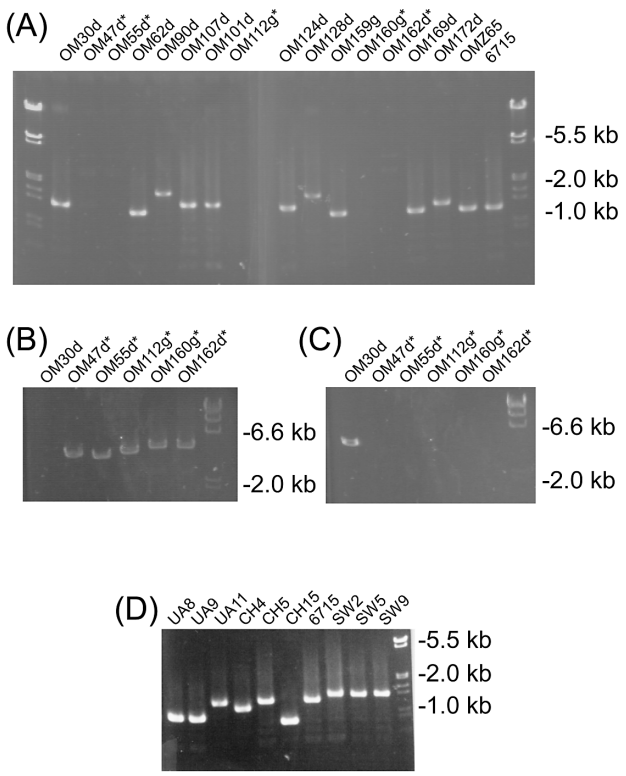
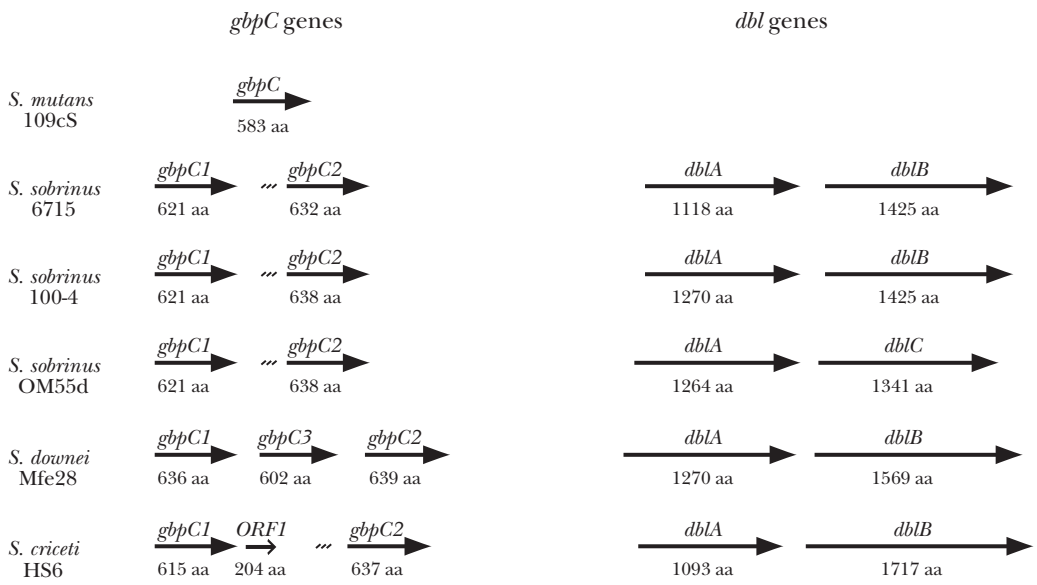


Fig. 1 Electrophoretic analyses of *dblB* and *dblC* PCR amplifications

(A) (D) *dblB* 5′ direct repeat regions amplified with primers, 5′-ACTAAATATGAAGTTGCAAAGTCATT-3′ and 5′- GTGATAATGATTTGGCTTGAGCT-3′. (B) extracellular domain-encoding regions of *dblC* gene amplified with primers, 5′-CACTCGAGGATGTCAGCAGTCAGGCTAATCCA-3′ and 5′-TCAAGTCTGCGGCAGGGTCTTTT-3′. OM30d were negative controls. (C) extracellular domain-encoding regions of *dblB* gene amplified with primers, 5′-ACCTCGAGATGAAACAGCAGGTAATTCCGC-3′ and 5′-ATGAATTCGTCTGTGGCAGAGTTTTTTCAGATGT-3′. *dblB* gene fragments were not amplified from *dblC*-positive strains in contrast to OM30d as positive control. (D) UA-, CH-, and SW-prefixed strains represent USA, China, and Sweden strains, respectively. Asterisks in (A)–(C) represent *dblA/dblC*-harboring strains.

conserved ORF sequence encoding a putative cation-transporting P-type ATPase, which was located 165 bp downstream from the termination codon of the *dblB* gene in strain 6715, started at 417 bp downstream from the termination codon of the *dblC* gene in OM55d. These data suggest that *S. sobrinus* strains harboring the *dblC* gene instead of the *dblB* gene may be present in natural *S. sobrinus* populations. To confirm this hypothesis, we performed PCR amplification with *dblB*- and *dblC*-specific primer sets in our *S. sobrinus* stock culture collection (OM-prefixed strains, except for OMZ65 in Fig. 1, were isolated from 3rd-year students of our dental college in 1978). The previously sequenced *S. sobrinus* *dblB* genes contained approximately 1.2-kb direct repeats near the 5′ region (5′DR). A *dblB*-specific primer set was designed to amplify these 5′DRs. The *dblB* gene was not amplified in 5 of 17 strains examined and the other 12 strains as well as previously reported strain 100-4 were *dblB*-positive (Fig. 1A). In addition, the sizes of the 5′DR indicated a diversity probably resulting from different numbers of the repeating unit (Fig. 1A). Furthermore, the *dblC* gene was amplified from the 5 *dblB*-negative strains with a PCR primer set designed for fragments corresponding to the extracellular domain of the OM55d

Table 1 *gbpC/dbl* gene arrangements among mutans streptococci



Shaded lines represent nucleotide sequence regions similar to *gbpC3* 3′ sequence region of *S. downei*.

DblC protein without the signal sequences (Fig. 1B). The results suggest that the 5 strains including OM55d contained the *dblC* gene instead of the *dblB* gene. We have previously obtained chromosomal DNA samples isolated from USA-, China-, and Sweden-*S. sobrinus* strains (3 strains each) by Dr. Caufield at New York University. All of these were *dblB*-positive (Fig. 1C) and *dblC*-negative strains. Gene arrangements concerning the *gbpC1*, *gbpC2*, *dblA*, and *dblB*/*dblC* genes in *S. sobrinus* are summarized in Table 1.

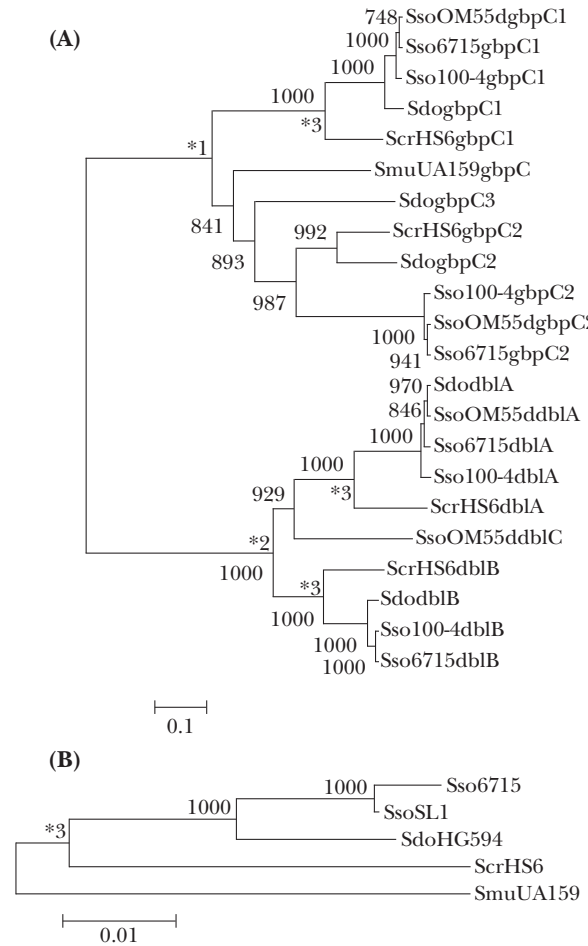
2. Amplification and nucleotide sequences of *gbpC/dbl* gene homologues in *S. downei* strain Mfe28

Regions corresponding to the *gbpC1*, *dblA*, and *dblB* genes of *S. downei* strain Mfe28 were amplified and sequenced with the primers used to determine these gene regions of *S. sobrinus*, except for sequencing several minor regions in the *gbpC1* gene, for which we designed new primers specific for *S. downei* nucleotide sequences. Nucleotide sequence identities of the *gbpC1*, *dblA*, and *dblB* genes between *S. downei* and *S. sobrinus* were respectively 91.2, 98.7, and 96.9%. Although the nucleotide sequence identity of the *gbpC1* gene between these two species was slightly lower than those for the *dblA* and *dblB* genes, the identity of a ~1,500-bp upstream region of the *gbpC1* gene was 97.1%, which corresponds to those for the *dblA* and *dblB* genes. A region downstream from the divergent 3′ portion of the *gbpC1* gene was therefore obtained by PCR-based genome walking and nucleotide sequencing revealed two other ORFs similar to the *gbpC2* gene. The second ORF was more similar (67% identity) to the *gbpC2* gene of *S. sobrinus* than the first ORF (54.5% identity). Therefore, these two ORFs were respectively designated as the *gbpC3* and *gbpC2* genes. Gene arrangements of the *gbpC/dbl* gene homologues in *S. downei* strain Mfe28 are also depicted in Table 1.

3. Amplification and nucleotide sequences of *gbpC/dbl* gene homologues in *S. criceti* strain HS6

S. criceti exhibits significant dextran-depen-

Table 2 Phylogenetic trees of *gbpC/dbl* genes (A) and 16S ribosomal RNA genes (B)



Phylogenetic analysis was performed using DDBJ ClustalW program with default setting, except for Bootstrap-On. Trees were drawn with TreeExplore program (http://evolgen.biol.metro-u.ac.jp/TE/). Bars represent genetic distances.

dent aggregation similar to *S. sobrinus* and *S. downei*[4]. Therefore, we presumed that *S. criceti* might have *gbpC/dbl* gene homologues similar to the latter organisms. Amplification with a primer set (YF2 and YR2) with which the *S. sobrinus gbpC* partial fragments could be amplified revealed approximately 0.3, 0.7, and 4-kb bands. The 0.3 and 0.7-kb fragments were excised from the gel, purified, and directly sequenced. The 0.3 and 0.7-kb sequences were highly similar to the corresponding sequences within the *S. sobrinus gbpC2* and *dblA* genes, respectively. The entire *gbpC2* and *dblA-dblB* gene sequences in *S. criceti* were determined following PCR-based genome walking (Kojima, Y., IADR abstract, 2009, http://iadr.confex.com/iadr/2009miami/webprogram/Paper115741.html). The *gbpC1* gene was not detected 1.4 kb upstream or 1.7 kb downstream from the *gbpC2* gene on the *S. criceti* chromosome. However, the *S. criceti* genome project (Project ID: 51461) has recently been in progress and nucleotide sequence data are now available

with the BLAST homology search. When the *S. sobrinus gbpC1* gene was searched against this database, a similar sequence was detected 1,790 bp upstream from the initiation codon of the *gbpC2* gene. Within this intergenic 1,790 bp region, one large open leading frame (ORF1 depicted in Table 1) encoding a 204-amino acid protein was detected. However, this amino acid sequence was not similar to any part of the GbpC protein. The *dblB* gene was located 356 bp downstream from the *dblA* termination codon on the same strand and encoded the largest DblB protein among these three species. Gene arrangements of the *gbpC/dbl* gene homologues in *S. criceti* strain HS6 are also depicted in Table 1.

4. Phylogenetic analysis of GbpC/Dbl protein homologues

Sequence similarities of GbpC/Dbl protein homologues between two MS species were higher than those between two of these homologues within a species. For example, the similarities of DblA (71.4%) or DblB (80.2%) between *S. sobrinus* and *S. criceti* were higher than similarity between DblA and DblB within *S. sobrinus* (46.1%) or *S. criceti* (45.6%). Phylogenetic analysis was performed with 21 *gbpC/dbl* gene homologue sequences together with 16S ribosomal RNA genes of MS using the DDBJ ClustalW program. The unrooted phylogenetic tree constructed for these genes is shown in Table 2. The *gbpC1*, *gbpC2*, *dblA*, or *dblB* gene was classified in a different monophyletic cluster, irrespective of MS species. In addition, the branching patterns of the *gbpC1* and *dblB* genes were very similar to that of the 16S rRNA genes. These branch shapes were similar to that of the glucosyltransferase gene *gtfI* from these species[13]. These results suggest that the divergence of *gbpC/dbl* genes occurred in a common ancestral species of these MS. A node with *1 indicates divergence of the *gbpC1* and *gbpC/ gbpC2/ gbpC3* genes, and a node *2 indicates divergence of the *dblA/dblC* and *dblB* genes. In contrast, node *3 represents divergence of *S. criceti* and other MS species.

5. Multiple alignment of *gbpC1-gbpC2* intergenic regions

The entire *gbpC* gene regions from *S. sobrinus*, *S. criceti*, and *S. downei* were analyzed by the ClustalW program at DDBJ. In addition to the highly conserved alignment of the *gbpC1* and *gbpC2* gene regions of these three organisms, a 3′ region of the *S. downei gbpC3* gene was aligned with several stretches of intergenic regions between the *gbpC1* and *gbpC2* genes from both *S. sobrinus* and *S. criceti*, as indicated in Table 3. The regions between the *gbpC1* termination codons and the *gbpC2* initiation codons in *S. sobrinus*, *S. criceti* and *S. downei* were respectively 686, 1,790, and 3,159 bp (in which the *gbpC3* gene was located). These results suggest that a portion of the *gbpC3* gene region harbored by an ancestral species of these three species may have been deleted from the chromosomes of *S. sobrinus* and *S. criceti* following species divergence, in contrast to *S. downei*.

Discussion

We previously identified the tandemly located *gbpC1-gbpC2* and *dblA-dblB* genes in *S. sobrinus*[10,11]. Paralogous genes are often organized in a tandem localization following unequal crossover recombination with highly similar sequences between the two daughter DNAs during replication in a species. Orthologous genes are those in different species that are derived from a common ancestor (http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/Orthology.html). However, we could not determine whether these two homologous gene pairs were paralogous in this study, as no similar genes were detected in multiple species related to *S. sobrinus* using the BLAST program of the NCBI against the international DNA database (EMBL/GenBank/DDBJ). Therefore, we initially intended to identify *gbpC/dbl* genes in *S. criceti* and *S. downei* as related species. Phylogenetic analysis indicated that the *gbpC1*, *gbpC2*, *dblA* and *dblB* genes from *S. sobrinus*, *S. criceti* and *S. downei* are positioned in clusters. Therefore, these

Table 3 Multiple alignment of *gbpC3* 3′ region with regions upstream from *gbpC2* genes in *S. criceti* and *S. sobrinus*

```
Sso6715gbpC1/2   ----AGGACCTTAAAAATGACAGCAGATGAGA---------------AAATTTTGGCCTT
ScrgbpC1/2       ----AGGAAATCAGAGATGACAGCAGATGAAA---------------AAATTTTAGCTTT
SdogbpC1/2/3     GCACAAGAACCTGGTGACTGTAGCACAGGAAGCTCCTCAAAAGCCGCAGACTCTAGAAAA
                       * **          *     **** * **                    * * * * *

Sso6715gbpC1/2   GATT---------AAACCAG----AATATCTGGAAAGGGT--------TCCGAGAA----
ScrgbpC1/2       GGT---------AAAACCAG----AATATATGGAAAGAAT--------TCCTAAAA----
SdogbpC1/2/3     GGCTCAGCCACAAAAACCAGCCCAAGTATGGGAAATGAAGACTAGCGGCCCTCAAGCTAG
                 *              *******     * ***   * ** *             **    *

Sso6715gbpC1/2   ---------TGTTTCGTGGTCATGCTACAAAGACAACTGTAAAAAAAAAGATTGCTCAAG-
ScrgbpC1/2       ---------TGTTTCGGGGACATGCCACCAAGGCAACGATAAAAAA--GATTGCTCAGG-
SdogbpC1/2/3     CCAACCAGCTACTAAGAGTGCTAGCCTTCCAGAAACCGGTGACAGA--CCTAGCTATGGC
                           *   *   * *   *   **       **   * *   * * * *     * ***    *

Sso6715gbpC1/2   ------------------AGTAT--AGACCTTTATGCTA--------------------
ScrgbpC1/2       ------------------AACATCCAGACCTTTATGCTA--------------------
SdogbpC1/2/3     CTTGCTGCCTTCGGTGCGGGTATCCTAGCCTTTACCCTAGCAACCACCCTGGCTACCGCT
                                   **       ******   ***

Sso6715gbpC1/2   AGTC----GGAGGT--GGCGGGGGA--TCTGCCAGATGAAG-------CGACTCAGGAGT
ScrgbpC1/2       AAGC----AGAGGA--AGCTGGGGA--ATTACCAGATGA--------CTTGGCTCAAGAAT
SdogbpC1/2/3     AAGCGAAAAGAGGACTAGATAGGGGCCGTCATCAACTGGAGAAAATCCTAGTGCTGGGCT
                 *   *       ****     *    ***          **   **                 *   *   *

Sso6715gbpC1/2   TA----TCAATCATTATCAACGGTATCTTTGAGGTT---AAGATAAAAAAGCATAATTTC
ScrgbpC1/2       T---GTCTAGC-ATTATCAATGGTATCTTTGAAGCT---AAGATGAAAAAGCACAATTTC
SdogbpC1/2/3     TATAGTTCAGCTGCCCTCACTCCTTATCGTCAAGCTTTCAGTATTCTGCTGGAAGCTTTT
                 *          *          ***     *       * * * *    *   **        * *    ***

Sso6715gbpC1/2   TAGT----------------AAAT----------TTAGCATTAGCGGT----------
ScrgbpC1/2       TAAATCTTTATTTTGCTTTGAAAGT--CAGTTCAGTTTGGGACTGGTTTTT-----TTAT
SdogbpC1/2/3     TGATTTCCTATTTTGCTAGAAAAGTGATAGACTAGGTCAAAAAAAGAGATGGGAGGTTAT
                 *                        ** *             *     *    *    *
```

Regions between *gbpC1* termination codons and *gbpC2* initiation codons in *S. sobrinus*, and *S. downei* were respectively 686, 1,790, and 3,159 bp (in which *gbpC3* gene was located), and are respectively numbered from nucleotides immediately following *gbpC1* termination codons. Aligned *gbpC1/gbpC2* intergenic regions indicated in this figure are: 215–479 in *S. sobrinus*, 1221–1516 in *S. criceti*, and 2286–2704 (*gbpC3* 3′:2286–2539) in *S. downei*. Box represents termination codon of *S. downei gbpC*.

gbpC/dbl genes from these three species appeared to be orthologous and derived from a common ancestor. In addition, it is of interest that *S. downei* possessed three *gbpC* genes distinct from those in two other species. Multiple alignment of *gbpC1-gbpC2* intergenic regions analyzed by the ClustalW program at DDBJ suggested that an ancestor species of these three species harbored the *gbpC3* gene rather than *S. downei* acquiring the *gbpC3* gene from another unknown species by horizontal transfer.

It is also of interest that *S. sobrinus* populations can be classified into two groups relative to *dbl* gene organization. One harbors the *dblA-dblB* tandem genes and the other has the *dblA-dblC* tandem genes. Downstream regions from the *dblB* and *dblC* gene regions are conserved between the two groups of *S. sobrinus* strains as described above. These results suggest that an ancestor species of these three species might also have possessed a *dblA-dblB-dblC* cluster similar to the *gbpC1-gbpC3-gbpC2* genes. The ratio of *dblA-dblC* harboring strains to *dblA-dblB* harboring strains was 5 to 12 in our stock culture collection and strain SL1

was the sole *dblA/dblC* harboring strain among the reference strains (OMZ65, 6715, B13) and each of the three China, USA, and Sweden strains. Therefore, geographically biased distribution of the two groups may be possible, although sample numbers may be too small.

Recently, new organisms classified as MS have been reported in other animals, including pig, bat, and wild boar[14,15], although their pathogenicity in their hosts remains to be fully characterized. It will be of interest to determine which types of *gbpC/dbl* variation exist in these species.

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