Molecular characterization of the dextran-binding lectin B gene *dblB* of *Streptococcus criceti* in *Streptococcus mutans* strain GS-5 with mutations in both *gbpC* and *spaP* genes

Haruki Tamura*, Arisa Yamada and Hirohisa Kato

Division of Bioregulatory Pharmacology, Department of Pharmacology, Iwate Medical University,
2-1-1 Nishitokuta, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan

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Streptococcus mutans, a cariogenic agent, has a glucan-binding protein gene, *gbpC*, and *S. criceti* possesses four *gbpC* homologs, including *dblA* and *dblB*, as does *S. sobrinus*. The *S. criceti* *dblB* gene encodes a 1,717-amino-acid protein having two repetitive alanine-rich and proline-rich regions and an LPXTG motif, which is recognized by the sortase SrtA, near the C terminus. Reverse transcription-PCR analysis indicated no cotranscription of the *dblA* and *dblB* genes of *S. criceti*. As we could not obtain a *dblB* mutant of *S. criceti*, the *dblB* gene was characterized in *S. mutans* strain GS-5, which has genetic mutations in both *gbpC* and *spaP* genes and shows an inability to agglutinate triggered by dextran. A dextran-induced agglutination assay showed that *S. mutans* cells carrying *dblB* agglutinated in the presence of dextran. A hydrophobicity assay showed that the cells containing *dblB* were hydrophobic. A biofilm formation assay showed that the *dblB* gene was associated with biofilm formation by cells cultivated in brain heart infusion broth supplemented with glucose and maltose, but not sucrose. Nucleotide sequence analysis of the *S. criceti* strains studied revealed a frameshift mutation in the *srtA* gene encoding sortase, but intact *dblA* and *dblB* genes were found in dextran-induced agglutination-negative strains, whereas intact *dblA*, *dblB* and *srtA* genes were found in dextran-induced agglutination-positive strains. These results suggest the cell-surface localization of *dblA* and *dblB* gene products by SrtA and the responsibility of *dblB* for dextran-induced agglutination, cell-surface hydrophobicity and biofilm formation in *S. criceti*.

Key words: biofilm formation, cell surface hydrophobicity, dextran-induced agglutination, mutans streptococci

INTRODUCTION

Streptococcus mutans is a cariogenic agent in humans (for review, see Colby and Russell, 1997) and possesses cell-surface proteins such as glucan-binding protein C (GbpC) (Sato et al., 1997) and SpaP (also known as antigen I/II, PAc, P1 and antigen B) (for review, see Brady et al., 2010), which contain an LPXTG motif recognized by sortase SrtA for anchorage to the cell wall (Igarashi, 2004). SpaP contains two repetitive domains, an alanine-rich A region and a proline-rich P region, and is involved in binding to salivary glycoproteins (Sciotti et al., 1997) and in cell-surface hydrophobicity (Murakami et al., 1997). GbpC is responsible for dextran-dependent agglutination in *S. mutans* grown under stress conditions such as at a subinhibitory concentration of tetracycline,

4% ethanol, or 42°C incubation (Sato et al., 1997). Agglutination also occurs through the transcriptional regulator IrvA, which promotes agglutination via not only GbpC but also an increase of SpaP (Zhu et al., 2009). *S. mutans* GS-5 fails to agglutinate in the presence of dextran owing to a nonsense mutation in the *gbpC* gene (Sato et al., 2002). Furthermore, a frameshift mutation in the *spaP* gene has been found in one strain of GS-5 (Murakami et al., 1997), but intact *spaP* has been identified in other strains of GS-5 (Sato et al., 2002; Biswas and Biswas, 2012).

Streptococcus criceti is a cariogenic organism rarely found in humans and is a member of the mutans streptococci, including *S. mutans* and *S. sobrinus* (for review, see Colby and Russell, 1997). *S. criceti* strain HS-6^T contains four *gbpC* homologs encoding LPXTG-containing proteins, namely *gbpC1*, *gbpC2*, *dblA* and *dblB* genes; the *gbpC1* and *gbpC2* genes are tandemly located in one locus

* Edited by Kazuhiro Kutsukake

* Corresponding author. E-mail: htamura@iwate-med.ac.jp

and the *dblA* and *dblB* genes in another, as occurs in *S. sobrinus* strain 6715 (Kojima et al., 2012). Dextran-induced agglutination is a characteristic of *S. criceti*, similar to *S. sobrinus* (Drake et al., 1988). Among the four *gbpC* homologs in *S. sobrinus* strain 6715, the dextran-binding lectin DblB is most responsible for dextran-dependent cell agglutination (Sato et al., 2009). However, it is not known how dextran-induced agglutination occurs in *S. criceti*. Furthermore, dextran-induced agglutination ability varies in *S. criceti* strains: agglutination-positive strains are HS-6^T and OMZ 61, and agglutination-negative strains are E49 and HS-1 (Tamura et al., 2012). *dblA* and *dblB* genes encoding 1,093- and 1,717-amino-acid proteins, respectively, have been identified in strain HS-6^T (Kojima et al., 2012), but nucleotide sequences of the *dblA* and *dblB* genes have not been determined in strains E49, HS-1 or OMZ 61.

As we could not obtain a *dblB* mutant of *S. criceti*, we characterized the *dblB* gene of *S. criceti* in *S. mutans* using a dextran-induced agglutination assay, a cell-surface hydrophobicity assay and a biofilm formation assay. Presuming that gene mutations of *dblA*, *dblB* and/or *srtA* might occur in dextran agglutination-negative strains of *S. criceti*, we sequenced all three genes in three strains of *S. criceti* and found an *srtA* gene mutation in the two agglutination-negative strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions All strains and plasmids are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani broth (Difco Laboratories) at 37°C. Streptococcal cells were maintained

in brain heart infusion (BHI) broth (Difco Laboratories) in an anaerobic jar at 37°C. To select for transformants, spectinomycin was added to the broth for *E. coli* (50 µg/ml) and streptococci (500 µg/ml) as needed. For the biofilm formation assay, streptococcal cells were grown in a 5% CO₂ incubator at 37°C.

DNA preparation, manipulation and transformation Bacterial DNA was purified using the Wizard Genomic DNA Purification Kit (Promega). Plasmid DNA was isolated using NucleoSpin Plasmid (Macherey-Nagel GmbH). To obtain *dblA*, *dblB* and *srtA* genes from *S. criceti* and *gbpC*, *spaP* and *srtA* genes from *S. mutans*, PCR was performed using *TaKaRa LA Taq* DNA polymerase (Takara Bio) with the primers listed in Table 2. The PCR conditions were as follows: 94°C for 1 min; 35 cycles of 94°C for 10 sec, 51 to 57°C for 15 sec and 72°C for 5 to 8 min; 72°C for 10 min. Agarose gel electrophoresis was performed to separate PCR products. Competent cells of *E. coli* strain Stellar were transformed in accordance with the supplier's protocol (Clontech Laboratories). *S. mutans* was transformed using heat-inactivated horse serum as described previously (Perry et al., 1983).

DNA sequence analysis DNA sequencing of PCR products was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). DNA sequence analysis and signal peptide prediction were performed with the computational package GENETYX ver.11 (Genetyx). Sequence alignments were generated using Clustal W2 (Larkin et al., 2007). Nucleotide sequence data

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i> Stellar	<i>F⁻ ara Δ(lac-proAB) [Φ80d lacZΔM15] rpsL(str) thi Δ(mrr-hsdRMS-mcrBC) ΔmcrA dam dem</i>	Clontech Laboratories
<i>S. criceti</i>		
HS-6	Type strain; dextran agglutination-positive	American Type Culture Collection
E49	Dextran agglutination-negative	Laboratory collection
HS-1	Dextran agglutination-negative	Laboratory collection
OMZ 61	Dextran agglutination-positive	Gift of Hidehiko Mukasa
<i>S. mutans</i>		
GS-5	Wild type	Laboratory collection
GS-5P	Transformant of GS-5 with pDL278; Spc ^r	This study
GS-5B	Transformant of GS-5 with pDL278B; Spc ^r	This study
Plasmids		
pDL278	Shuttle vector; Spc ^r	LeBlanc et al. (1992)
pDL278B	pDL278 carrying <i>S. criceti</i> <i>dblB</i> gene; Spc ^r	This study

^a Abbreviations: Spc^r, spectinomycin-resistant.

Table 2. Primers used in this study

Designation	Sequence (5' to 3') ^a
For RT-PCR of <i>dblA</i> gene of <i>S. criceti</i>	
AF	GAAACTGAGGCGAAGGTCCA
AR	TAGGCACCTTTAGCTTGGGC
For RT-PCR of <i>dblB</i> gene of <i>S. criceti</i>	
BF	CTGATGCTCGCAATGCTGAC
BR	GTTTGCGTGTTTTGCGCTTC
For RT-PCR of <i>dblA/dblB</i> region of <i>S. criceti</i>	
ABF	ACCCACCAAAATCTGTACAACC
ABR	TCACTTGACTCGTCACAGTTGTC
For cloning of <i>dblB</i> gene of <i>S. criceti</i>	
IndblBScF	gcaggcatgcaagcttGCCGGTATTATTGCCTTCTCCATC
IndblBScR	tgattacgccaaagcttGTAACCGCCTTCTTTCTAATTACACCT
For amplification of <i>dblA</i> gene of <i>S. criceti</i>	
dblAScF	TGGTAAGAAGTCTATGGCCTACA
dblAScR	GATCATGTGCTGCTTTGGGTAGA
For amplification of <i>dblB</i> gene of <i>S. criceti</i>	
dblBScF	GCCGGTATTATTGCCTTCTCCA
dblBScR	GTAACCGCCTTCTTTCTAATTACACC
For amplification of flanking region of <i>srtA</i> gene of <i>S. criceti</i>	
srtAScF	ACTTGCCAGTGAATACCCAAC
srtAScR	CTGACGTCTTGGTGGCAACT
For amplification of <i>gbpC</i> of <i>S. mutans</i>	
gbpCSmF	GCAACTTTATCCTCAGTCGTTTC
gbpCSmR	CCTTGTGCAGCATCTACCA
For amplification of <i>spaP</i> of <i>S. mutans</i>	
spaPSmF	GAAAGACAACGACAGTAGAGG
spaPSmR	AACGTTATTGGTCACGTCAA
For amplification of flanking region of <i>srtA</i> gene of <i>S. mutans</i>	
srtASmF	TTAACGAGAGCGAAAGCGTAA
srtASmR	CGGATTGTTAGCCTTAACCTACT

^a Primers were designed from the nucleotide sequences of *S. criceti* strain HS-6^T (DDBJ accession numbers AEUV02000002 and AB557645; Kojima et al., 2012) and *S. mutans* strain GS-5 (DDBJ accession number CP003686; Biswas and Biswas, 2012).

The sequence homologous to the shuttle vector pDL278 (LeBlanc et al., 1992) for in-fusion cloning is shown in lower case. *Hind*III sites are underlined.

reported are available in the DDBJ/EMBL/GenBank databases under accession numbers AB742519, AB751198–AB751200, AB819737 and AB910261–AB910263.

RNA isolation and RT-PCR Total RNA was isolated with TRI reagent (Sigma-Aldrich) and contaminating DNA was then degraded with amplification-grade DNase I according to the manufacturer's instructions (Sigma-Aldrich). RNA (1 µg) was treated with a random primer and GoScript reverse transcriptase (Promega) to generate cDNA. Reverse transcription-PCR (RT-PCR) was performed with *TaKaRa Taq* DNA polymerase (Takara Bio) and the primers listed in Table 2, under the following conditions: 94°C for 1 min; 40 cycles of 94°C for 10 sec, 57°C

for 15 sec and 72°C for 1 min; 72°C for 3 min. The authenticity of the amplified fragments was confirmed by direct sequencing.

Construction of plasmid containing the *dblB* gene and bacterial strains Primers IndblBScF and IndblBScR, listed in Table 2, were used for PCR amplification of the region from 419 bp upstream of the *dblB* start codon to 319 bp downstream of the *dblB* termination codon. The resulting fragment, amplified by PrimeSTAR MAX DNA polymerase (Takara Bio), was cloned into the *Hind*III site of the *E. coli-Streptococcus* shuttle vector pDL278 (LeBlanc et al., 1992) using the IN-Fusion HD Cloning System (Clontech Laboratories) to generate

pDL278B. The nucleotide sequence of the *dblB* gene of the plasmid was verified by DNA sequencing. *S. mutans* strain GS-5 was transformed with plasmids pDL278 (LeBlanc et al., 1992) and pDL278B, resulting in strains GS-5P and GS-5B, respectively. The existence in these strains of a region containing the plasmid-borne spectinomycin resistance gene was confirmed by PCR.

Dextran-induced agglutination assay The agglutination assay was performed by modifying a method described previously (Tamura et al., 2012). Bacterial cells cultured anaerobically at 37°C for 16 h in BHI broth were washed three times with phosphate-buffered saline (PBS). Cell suspensions prepared in polypropylene tubes were adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0. For the assay, cell suspensions transferred to glass tubes were or were not supplemented with dextran (200 µg/ml) and mixed for 20 sec. After the indicated time, agglutination was assessed visually.

Hydrophobicity assay Surface hydrophobicity of streptococcal cells was determined by their adsorption on hexadecane as described by Koga et al. (1990) with some modifications. Briefly, the cells were grown at 37°C for 16 h in BHI broth, and then washed three times and suspended in PBS to an OD₆₀₀ of 1.0. Hexadecane was added and the suspension was mixed for 1 min. After incubation for 15 min, turbidity of the aqueous phase was measured at 600 nm. Hydrophobicity is represented as the percentage of adsorbed cells.

Biofilm formation assay Biofilms produced in flat-bottomed 96-well polystyrene cell culture plates (TPP Techno Plastic Products) were evaluated by slightly modifying the method described previously (Yamada et al., 2009). Briefly, bacterial cells (1.0×10^4 CFU) were cultured in BHI broth, with or without 1% glucose, sucrose or maltose, at 37°C for 16 h under 5% CO₂ in air. Biofilms were stained with 1% crystal violet solution and then washed three times and air-dried. The adhered crystal violet was solubilized with ethanol and the optical density at 595 nm (OD₅₉₅) was determined.

RESULTS

RT-PCR analysis of *dblA* and *dblB* genes Nucleotide sequences of the *dblA* and *dblB* genes of *S. criceti* HS-6^T were retrieved from DDBJ/EMBL/GenBank databases (accession number AB557645; Kojima et al., 2012). As *dblA* and *dblB* genes are arranged in tandem (Fig. 1A), their expression was examined by RT-PCR. PCR products generated with primers for the *dblA* (197 bp) and *dblB* (295 bp) genes were obtained with reverse transcription and genomic DNA of *S. criceti* HS-6^T (Fig. 1B), whereas products for the *dblA/dblB* region (807 bp) were

Fig. 1. Transcriptional analysis of the *dblA/dblB* genes of *S. criceti*. (A) Schematic representation of the *dblA* and *dblB* region of *S. criceti* strain HS-6^T. Arrows and arrowheads indicate open reading frames (ORFs) and the positions of primers used for RT-PCR analysis, respectively. Numbers inside and between arrows indicate the size of ORFs and intergenic regions, respectively, in base pairs. Sequence data are from DDBJ accession number AB557645 (Kojima et al., 2012). (B) RT-PCR analysis in a 1.5% agarose gel. The sizes of PCR products and representative DNA standards are shown on the right and left, respectively. Lane M, size marker; lane 1, RT-PCR mixture; lane 2, negative control without RT; lane 3, positive control of PCR with genomic DNA of HS-6^T. The results are representative of three separate experiments.

obtained only with genomic DNA. Thus, expression of the *dblA* and *dblB* genes was found, but their cotranscription was not observed.

Structural characterization of DblB The predicted translational product of the *dblB* gene, DblB, is a protein of 1,717 amino acids with an estimated molecular weight of 182,703. A signal peptide sequence containing a YSIRK-G/S motif (Bae and Schneewind, 2003) was found in the N-terminal region (residues 1–36: MEKNAQRF-SIRKYSFGAASVLLGTAVFVLSAPT VLA, underlined letters indicating the motif sequence). An LPXTG motif for sorting on the cell surface by sortase (Schneewind et al., 1993), LPQTG, was found in the C-terminal region (residues 1,681–1,685). These results suggest that DblB is a cell-surface protein sorted by sortase SrtA.

DblB contains two repetitive regions, termed the A and P regions (Fig. 2A). The A region is rich in alanine (ranging from 16 to 18 per repeat unit, namely, 32.7–

36.7% occupied) and contains ten repeating units (A1–A10) of a 49-residue sequence with 26 residues completely conserved in all the repeats (Fig. 2B). The P region is rich in proline (8 to 11 per repeat unit; 33.3–45.8% occupied) and includes 11 repeat units (P1–P11) of a 24-residue sequence with 9 residues completely conserved in all the repeats (Fig. 2C). Two sequences similar to the repeat unit were found around the P region and were designated as PreP and PostP.

Sequence analysis of *gbpC*, *spaP* and *srtA* genes of *S. mutans* strain GS-5 As transformants of *S. criceti* cells could not be obtained, we attempted to characterize

the *dblB* gene in *S. mutans*. To minimize the effects of *S. mutans* cell-surface proteins such as GbpC and SpaP on experiments, we chose strain GS-5, a dextran agglutination-negative strain (Sato et al., 2002). As three laboratories have reported two variants of strain GS-5, a *spaP*-mutant strain (Murakami et al., 1997) and a *gbpC*-mutant and *spaP*-intact strain (Sato et al., 2002; Biswas and Biswas, 2012), we verified the *gbpC*, *spaP* and *srtA* genes of strain GS-5 in our laboratory. Nucleotide sequence analysis of *gbpC* and *spaP* genes (assigned DDBJ accession numbers AB742519 and AB819737, respectively) showed that a nonsense mutation in *gbpC* occurs at codon 65 and an insertion mutation in *spaP*

Fig. 2. Structural characteristics of DblB of *S. criceti*. (A) Schematic representation of DblB. The signal peptide sequence (residues 1–36), alanine-rich repeat region (A region, residues 265–754) and proline-rich repeat region (P region, residues 1,325–1,588) are indicated. Locations of PreP and PostP flanking the P region and an LPXTG motif (residues 1,681–1,685) are shown. (B) The A region of DblB. Ten repeating units, A1 to A10, of a 49-residue alanine-rich sequence were found. Multiple sequence alignment was constructed by Clustal W2 (Larkin et al., 2007). Asterisks and periods indicate fully conserved and weakly similar residues, respectively. (C) Amino acid sequence of DblB around the P region. Eleven tandem repeats, P1 to P11, of a 24-residue proline-rich sequence were found in the P region and are flanked with sequences PreP and PostP, which are similar to the repeat unit. Alignment of the 11 repeat units (P1–P11) was performed using Clustal W2 (Larkin et al., 2007). Asterisks and periods indicate completely conserved and weakly similar residues, respectively.

Fig. 3. Dextran-induced agglutination assay. (A) Cell suspensions adjusted to an OD₆₀₀ of 1.0 were supplemented with dextran (200 µg/ml), mixed, and then incubated at ambient temperature for 30 min. The wild-type strain GS-5, GS-5P (GS-5 harboring an empty vector) and GS-5B (GS-5 harboring pDL278B carrying the *dblB* gene) were used. (B) GS-5B cell suspensions adjusted to an OD₆₀₀ of 1.0 were or were not supplemented with dextran (200 µg/ml), mixed, and then incubated at ambient temperature for 5 min. From the left: cell suspensions before the addition of dextran and vortexing, cell suspensions without dextran after vortexing, and cell suspensions after the addition of dextran and vortexing. Data are representative of three separate experiments.

occurs at codon 1,157, indicating that our strain is a *gbpC*- and *spaP*-mutant strain. The predicted translational products of the *gbpC* and *spaP* genes, consisting of 64 and 1,158 residues, respectively, are truncated and lack an LPXTG motif (Schneewind et al., 1993). The nucleotide sequence of the 5,173-bp region containing the *srtA* gene in our strain is identical to that of the genome-sequenced strain (DDBJ accession number CP003686) (Biswas and Biswas, 2012).

Dextran-induced agglutination assay of *S. mutans* cells The result of the agglutination assay showed that GS-5 and GS-5P (GS-5 harboring an empty vector) cells did not agglutinate in the presence of dextran (Fig. 3A). In contrast, GS-5B, containing the *dblB* gene, was rapidly agglutinated by dextran and clumped cells adhered to the glass tube (Fig. 3A). GS-5B cells without dextran adhered to the glass surface above the water phase, whereas the cells with dextran agglutinated and clumped (Fig. 3B). These results confirmed that the agglutination was induced by dextran and was *dblB*-dependent.

Hydrophobicity assay of *S. mutans* cells Cell-surface hydrophobicity was analyzed using hexadecane. GS-5B cells carrying the *dblB* gene showed the highest hydrophobicity among the three cells studied (Fig. 4). This observation indicated that the *dblB* gene is involved in cell-surface hydrophobicity in *S. mutans*.

Biofilm formation assay of *S. mutans* cells Biofilms formed by GS-5, GS-5P and GS-5B cells were evaluated

Fig. 4. Cell-surface hydrophobicity assay. Surface hydrophobicity of GS-5, GS-5P and GS-5B cells was determined by their adsorption on hexadecane. The results express the means and SDs (error bars) of triplicate samples, and are representative of three separate experiments.

(Fig. 5). Although BHI broth is an enriched medium, all three cells formed less biofilm in BHI medium alone. GS-5 and GS-5P cells formed poor biofilm in BHI broth with glucose, sucrose or maltose. In contrast, GS-5B cells formed more biofilm in BHI broth with glucose or maltose, but poor biofilm with sucrose. These findings suggest that the *dblB* gene product contributes to biofilm formation in BHI broth with glucose and maltose.

Sequence analysis of the *dblA*, *dblB* and *srtA* flanking regions of *S. criceti* strains To test the hypothesis that mutations in the *dblA* and/or *dblB* gene(s) might be present in the dextran agglutination-negative strains

Fig. 5. Biofilm formation assay. GS-5, GS-5P and GS-5B cells were cultivated on polystyrene cell culture plates and in BHI broth with or without 1% additional glucose, sucrose or maltose. Biofilms were stained with 1% crystal violet and OD₅₉₅ values were measured. The results express the means and SDs (error bars) of quadruplicate samples in three independent experiments.

E49 and HS-1, the 9,554-bp sequences of the *dblA* and *dblB* genes of strains E49, HS-1 and OMZ 61 were determined (Fig. 6A). In all three strains, the *dblA* and *dblB* genes were arranged in tandem, as they are in *S. criceti* strain HS-6^T (accession number AEUV02000002). The nucleotide sequences of the *dblA* and *dblB* genes of strains HS-1 and OMZ61 (accession numbers AB751199 and AB751200, respectively) were identical to those of strain HS-6^T. Regarding strain E49, a substitution was found (AB751198): codon 1,244 of *dblB* in E49 was CTT (leucine), and those in HS-6^T, HS-1 and OMZ 61 were CTC (leucine). These results show that the *dblA* and *dblB* genes in strains E49 and HS-1 are intact.

Next, we conjectured that mutations in the *srtA* gene might be present in the dextran agglutination-negative strains, as the responsibility of the *srtA* gene for anchoring cell-surface proteins containing an LPXTG motif in *S. mutans* has been reported (Igarashi, 2004). To test this, the *srtA* genes and flanking regions were sequenced in strains E49, HS-1 and OMZ 61 (Fig. 6B). The 3,837-bp sequence of the *srtA* region of strain OMZ 61 (accession number AB910263) was identical to that of strain HS-6^T (AEUV02000002). A comparison of the 3,836-bp sequences of the *srtA* regions of strains E49 and HS-1 (AB910261 and AB910262, respectively) with those of strains OMZ 61 and HS-6^T showed that a single cytosine deletion in the *srtA* gene at position 392 had occurred in strains E49 and HS-1 (Fig. 6C). This deletion causes a frameshift at codon 131 and introduces a premature stop codon into the *srtA* gene (Ala131Valfs*2). SrtA of strains HS-6^T and OMZ 61 is a 258-amino-acid protein containing a cysteine-217 residue, which was predicted as necessary for sortase activity as cysteine-184 of *Staphylococcus aureus* SrtA (Ton-That et al., 2002) (Fig. 6D). SrtA of strains E49 and HS-1 is, thus, a 133-amino-acid protein

lacking a conserved cysteine residue that may be required for sortase activity, suggesting that LPXTG-containing proteins cannot anchor to the cell wall in strains E49 and HS-1.

DISCUSSION

Little is known about the molecules associated with dextran-induced agglutination in *S. criceti*. Furthermore, the roles of the *dblA* and *dblB* gene products in *S. criceti* are not understood. As both genes are tandemly situated in strain HS-6^T (Kojima et al., 2012), their expression was examined. In our transcriptional analysis, cotranscription of *dblA* and *dblB* genes was not detected, whereas both gene transcripts were observed. These findings suggest that their expression is differently regulated by unidentified molecules.

The *dblA* and *dblB* genes have been identified from *S. sobrinus* strains including 6715, *S. criceti* strain HS-6^T and *S. downei* ATCC 33748^T; DblA of *S. criceti* is the smallest protein (1,093 residues) and DblB of *S. criceti* the largest (1,717 residues) in the three related species (Kojima et al., 2012). DblB of *S. criceti* strain HS-6^T showed 72.0% and 79.3% identity, respectively, to DblB (1,425 residues) of *S. sobrinus* strain 6715 (Sato et al., 2009) and DblB (1,569 residues) of *S. downei* ATCC 33748^T (Kojima et al., 2012), indicating a greater resemblance between *S. criceti* strain HS-6^T DblB and that of *S. downei* ATCC 33748^T than that of *S. sobrinus* strain 6715.

Our results revealed that the primary sequences of DblB in *S. criceti* strain HS-6^T contained two sequence motifs, the YSIRK-G/S motif for signal peptide processing (Bae and Schneewind, 2003) and the LPXTG motif for cell-wall anchoring, which is conserved in cell-surface proteins (Schneewind et al., 1993). This suggests that DblB is anchored to the cell wall by SrtA, as are other LPXTG-containing proteins such as DblA, GbpC1 and GbpC2. Furthermore, DblB exhibited 48.3% identity with DblA, suggesting that DblA and DblB are functionally distinct cell-surface proteins. It is of note that DblB contained two repeating regions, the A and P regions, as in the *S. mutans* cell-surface protein SpaP (for review, see Brady et al., 2010). A and P regions are also found in DblBs of *S. sobrinus* strain 6715 and *S. downei* ATCC 33748^T. DblB of *S. sobrinus* contains six repeating units (A1–A6) of a 49-residue sequence in the A region (residues 266–559) and seven repeat units (P1–P7) of a 24-residue sequence in the P region (residues 1,129–1,296). DblB of *S. downei* contains eight repeating units (A1–A8) of a 49-residue sequence in the A region (residues 266–657) and nine repeat units (P1–P9) of a 24-residue sequence in the P region (residues 1,227–1,442). Of the three, the largest DblB of *S. criceti* contains ten repeating units of a 49-residue sequence in the A region and eleven repeat units of

a 24-residue sequence in the P region.

In this study, *dblB* of *S. criceti* was expressed in *S. mutans* strain GS-5, which was confirmed as a *gbpC*- and *spaP*-mutant strain. In two laboratories, GS-5 was reported as a *gbpC*-mutant and *spaP*-intact strain (Sato et al., 2002; Biswas and Biswas, 2012). As at least two genotypes exist in strain GS-5, care should be taken in experiments using this strain. Use of the *gbpC*- and *spaP*-mutant strains is appropriate to eliminate the effects of GbpC and SpaP on experiments of dextran-induced agglutination, cell-surface hydrophobicity, and biofilm formation. Indeed, loss of their functions in *S. mutans* has demonstrated that the *gbpC* mutant was dextran-induced agglutination-negative (Sato et al., 1997), and the *spaP* mutant was hydrophilic regarding the cell surface (Koga et al., 1990). In accordance with a previous observation of strain GS-5 (Sato et al., 2002), our GS-5 cells were unable to agglutinate. Furthermore, we showed that the *dblB* gene is responsible for dextran-induced agglutination in *S. mutans*. It is of note that cells harboring *dblB* adhered to the glass surface above the water phase when cells were mixed without dextran, suggesting that *dblB* gene products act as adhesins on abiotic surfaces in *S. mutans* cells. Interestingly, the cell-surface hydrophobicity of cells containing *dblB* increased markedly. This is similar to SpaP, which is important in surface hydrophobicity in *S. mutans* (Koga et al., 1990). In this study, we showed that *S. mutans* strain GS-5 cells formed less biofilm in BHI medium alone. The magnitude of biofilm formation in *S. mutans* strain GS-5 in this study was lower than that in *S. mutans* strain Xc (Yamada et al., 2009). *S. mutans* Xc cells possess a SpaP of approximately 190 kDa (Koga et al., 1989). Sequence analysis of the *spaP* gene in this study predicted that GS-5 cells produce a 1,158-amino-acid protein lacking the cell-wall-anchoring motif LPXTG. Indeed, it has been demonstrated that the *spaP* gene of GS-5 encodes a 1,158-amino-acid protein and that GS-5 produces extracellularly a 155-kDa SpaP (Murakami et al., 1997). Therefore, the poor biofilm formation of GS-5 cells may be attributable to the loss of SpaP on the cell surface. Furthermore, we showed that cells carrying the *dblB* gene could form biofilms in BHI broth supplemented with glucose and maltose, suggesting the involvement of *dblB* gene products in biofilm formation in *S. mutans*. In contrast, biofilm formation was poor in BHI with sucrose. In *S. mutans*, glucosyltransferases encoded by the *gtfB* and *gtfC* genes synthesize primary water-insoluble glucans from sucrose (Yamashita et al., 1993) and the *gtfD* gene product synthesizes water-soluble glucans (Hanada and Kuramitsu, 1989). Furthermore, it has been reported that the expression of *gtfB* and *gtfC* decreases in the presence of 2% sucrose in BHI broth, whereas *gtfD* expression increases (Fujiwara et al., 2002). This implies that the expression of genes involved in biofilm

formation may alter in the presence of carbohydrates. Therefore, the elimination of cells from biofilms by the wash process in the biofilm formation assay might account for the decreased expression of *gtfB* and *gtfC* and the enhanced *gtfD* expression. The mechanism(s) of the diverse effects of carbohydrates on biofilm formation remain to be studied. Taken together, these findings suggest that the *dblB* gene product acts as agglutinin and plays a role in biofilm formation in *S. criceti*.

Our previous work showed that dextran-induced agglutination-negative strains were *S. criceti* strains E49 and HS-1 and *S. sobrinus* OMZ 65 (Tamura et al., 2012). In other non-agglutinating strains of *S. sobrinus*, changes in the *dblB* gene have been identified: strain OMZ 176, which possesses truncated DblA (584 residues) and DblB (254 residues), has mutations comprising a single adenine nucleotide insertion in the *dblA* gene and a 736-nucleotide deletion in *dblB*; and strain B-13N, which produces DblA (1,270 residues) but lacks DblB, shows replacement of the *dblB* gene by an IS1548-like sequence (Sato et al., 2009). Conversely, these findings support the responsibility of the *dblB* gene for dextran-induced agglutination in *S. sobrinus*. In contrast, it remains to be elucidated why some strains of *S. criceti* could not exhibit dextran-induced agglutination. Our results showed that *srtA* was mutated in dextran agglutination-negative strains, whereas the *dblA* and *dblB* genes were intact. This finding suggests that SrtA was responsible for the ability of dextran-induced agglutination via LPXTG-containing proteins such as DblB, an explanation which is supported by a report that inactivation of the *srtA* gene inhibited dextran-induced agglutination by GbpC in *S. mutans* (Igarashi et al., 2004). However, we cannot exclude the possibility of causative genes of dextran-induced agglutination other than *dblB*. It has been reported that in *S. sobrinus* strain 6715, dextran-binding activities are observed in GbpC2 and DblA as DblB, but there is no activity in GbpC1 (Sato et al., 2007). Thus, it is presumed that GbpC2 and DblA, but not GbpC1, of *S. sobrinus* and probably *S. criceti*, are independently involved in dextran-induced agglutination.

In *S. sobrinus*, strain 6715 possesses *dblA* and *dblB* genes (Sato et al., 2009) and strains OM55d and SL1^T harbor *dblA* and a *dblA/dblB* homologous gene, *dblC*, instead of the *dblB* gene (Kojima et al., 2012). This suggests variations of *dblA/dblB/dblC* gene arrangements in *S. sobrinus*. In contrast, the four *S. criceti* strains studied possessed *dblA* and *dblB* genes, suggesting that the *dblA* and *dblB* regions are conserved in *S. criceti*. Furthermore, tandemly arranged genes, such as *gtfB* and *gtfC* in *S. mutans*, are considered to have replicated by tandem duplication (Hoshino et al., 2012). Regarding the *dblA* and *dblB* genes, orthologous genes were found in *S. sobrinus*, *S. downei* and *S. criceti* (Kojima et al., 2012). Thus, tandem duplication might have occurred in

the ancestor of the three species.

In conclusion, the *dblB* gene of *S. criceti* was characterized. Our results suggest that the cell-surface localization of *dblA* and *dblB* gene products is governed by SrtA and that the *dblB* gene is responsible for agglutination induced by dextran, cell-surface hydrophobicity and bio-film formation in *S. criceti*.

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