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Short communication

Inactivation of *srtA* gene of *Streptococcus mutans* inhibits dextran-dependent aggregation by glucan-binding protein C

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A sortase-deficient mutant of *Streptococcus mutans* was prepared by insertional inactivation of a sortase gene (*srtA*). The *srtA* mutant was defective in cell wall-anchoring of two surface proteins 200 and 75 kDa in size. A previous study has shown that the 200 kDa protein is a surface protein antigen PAc and that the sortase catalyzes cell wall-anchoring of PAc in *S. mutans*. In this study another surface protein 75 kDa in size was examined by immunologic and physiologic methods. Western blot analysis with a specific antiserum showed that the 75 kDa protein was a surface protein, glucan-binding protein C. The protein was overexpressed under a stress condition including a sublethal concentration of tetracycline. The *srtA* mutant cells also lost the ability of dextran-dependent aggregation. These results suggest that the *S. mutans* sortase mediates cell wall-anchoring of the glucan-binding protein C and dextran-dependent aggregation of this organism.

Key words: cell wall anchoring; dextran-dependent aggregation; GbpC; LPXTG motif; sortase; *Streptococcus mutans*

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Streptococcus mutans is a gram-positive oral bacterium and the principal etiologic agent of human dental caries (5, 14). The formation of plaque biofilm on the tooth surface by *S. mutans* is an important step in the progression of dental caries, and many factors responsible for biofilm formation have been reported (5, 22). One of these factors, a surface protein designated glucan-binding protein C (GbpC), is responsible for the dextran-dependent aggregation of *S. mutans* (25). Sequence analysis of the *gbpC* gene reveals a sorting signal at the C terminus of the protein (19, 25) composed of an LPXTG motif, a hydrophobic domain, and a charged tail (19). This structural feature strongly suggests that GbpC is a cell wall-anchored protein. However, the mechanism of cell wall-anchoring of the GbpC has not been clarified.

Sortase (SrtA) is a membrane-localized transpeptidase that covalently links a surface protein with a sorting signal to the cell wall (15, 19, 28). SrtA and surface proteins with the sorting signal have been found in many gram-positive bacteria (19, 20) and play an important role in the pathogenesis of gram-positive bacterial infections (3, 9, 16, 19). In our previous study, we determined the complete nucleotide sequence of the *srtA* gene of *S. mutans* GS5 and suggested that the *S. mutans* SrtA catalyzes cell wall-anchoring of a surface protein antigen PAc containing a C-terminal sorting signal (8).

In this study we examined whether the SrtA of *S. mutans* is involved in cell wall-anchoring of GbpC and in the dextran-dependent aggregation of this bacterium.

S. mutans 109c and 109cS (25) were grown in Todd Hewitt Broth (Difco Laboratories, Detroit, MI). *Escherichia coli* JM109 is routinely used as a plasmid host and grown in Luria–Bertani broth.

Genomic DNA of *S. mutans* was purified by ultracentrifugation in a CsCl-ethidium bromide density gradient as described previously (6). Plasmid was extracted by a Wizard miniprep purification kit (Promega, Madison, WI).

srtA-deficient mutants of *S. mutans* 109c and 109cS were prepared by insertional inactivation of the *srtA* gene as previously described (8). Briefly, a plasmid pUE119srt harboring an internal portion (119 bp) of the *srtA* gene and an erythromycin resistance gene was introduced into *S. mutans* cells (21), generating *srtA*-deficient mutants with acquired erythromycin resistance (8). Insertion of the pUE119srt in the *srtA* gene of *S. mutans* was confirmed by polymerase chain reaction and Southern

hybridization analyses (data not shown). Polmerase chain reaction was carried out under conditions described previously (6).

Whole cells were suspended in 1% sodium dodecyl sulfate – 1% 2-mercaptoethanol, heated at 100°C for 5 min and centrifuged as reported previously (8, 17). The resulting supernatant was used as the cell extract. The cell extract and the culture supernatant were analyzed by SDS-PAGE and Western blotting with rabbit anti-GbpC serum. Western blotting was carried out as described previously (7, 8). Anti-GbpC serum was prepared as previously reported (27).

The dextran-dependent aggregation of *S. mutans* cells was performed as described by Sato et al. (25). Strain 109cS and the mutant lacking SrtA of *S. mutans* cells were grown overnight in BTR-G broth with or without 0.18 μg of tetracycline/ml (24, 25) and dextran T2000 (100 mg/ml) was subsequently added to the tube. After swirling for a few minutes, the aggregation of *S. mutans* cells was observable with the naked eye. The tubes were incubated for 60 min after addition of dextran.

To examine surface proteins that are linked to the cell wall of *S. mutans* by SrtA, *srtA*-deficient mutants of *S. mutans* 109c were prepared by insertional inactivation of the *srtA* gene as reported previously (8), and protein profiles in the culture supernatants of the wild type strain 109c and the *srtA* mutant were compared. Two protein bands newly appeared in the culture supernatant of the *srtA* mutant with molecular sizes of 200 and 75 kDa, respectively (Fig. 1A, lanes 1 and 2). Recently, we showed that the 200 kDa protein is a surface protein antigen designated PAc and suggested that the PAc is anchored to the cell wall of *S. mutans* by SrtA (8). In this paper we describe another surface protein with a molecular size of 75 kDa. To identify the 75 kDa protein released in the culture supernatant of the *srtA* mutant, the molecular size was compared with that of five LPXTG proteins found in *S. mutans*: PAc (accession no. X14490, X17390), GbpC (accession no. D85031), wall-associated protein A (accession no. M37842), dextranase (accession no. D49430), and exo-β-D-fructosidase (accession no. U78296). After comparing their molecular sizes, the 75 kDa protein was designated as GbpC (25, 27). Western blot analysis revealed that anti-GbpC serum clearly reacted with the 75 kDa protein in the culture supernatant of the *srtA*-deficient mutant (Fig. 1B, lane 2). In the cell extract of the wild type, the 72 kDa protein with lower molecular size degradation products was detected (Fig. 1B, lane 3). The protein released in the supernatant of the *srtA* mutant (75 kDa) is bigger than the protein extracted from the wild type (72 kDa); the 75 kDa protein retains a C-terminal sorting signal with an LPXTG motif as this protein does not experience the anchoring process to the cell wall by SrtA in the *srtA*-deficient mutant. Therefore, it is thought that the 72 kDa protein is a wall-anchored form of GbpC, which corresponds to the molecular size (72 kDa) of GbpC extracted from a wall fraction of *S. mutans* by Sato et al. (27). In contrast, the 72 kDa protein and its degradation products apparently disappeared in the cell extract of the *srtA* mutant (Fig. 1B, lane 4). Thus, a mutation of the *srtA* gene of *S. mutans* resulted in the complete loss of surface attachment of GbpC.

As it is reported that expression of GbpC is elevated in cells grown under a variety of stress conditions including the addition of tetracycline, ethanol or xylitol (25, 26), the stress response of the proteins reactive with anti-GbpC serum was examined in *S. mutans* cells grown under a stress condition including a sublethal concentration of tetracycline. The protein bands reacted with anti-GbpC serum were clearly overexpressed under the stress conditions (Fig. 1B,C). Previous genetic information shows that the genome of *S. mutans* encodes five surface proteins bearing a C-terminal sorting signal: GbpC, PAc, Dex, FruA and WapA. In these surface proteins, it is reported that only GbpC is a stress protein (19, 25). In addition, our preliminary study showed that anti-GbpC serum did not react with recombinant PAc, Dex, FruA and WapA (data not shown). These results would support the assumption that the protein bands detected by anti-GbpC serum were stress protein GbpC.

Under conditions of stress, GbpC of *S. mutans* enables the cells to aggregate in the presence of dextran (25). Figure 2

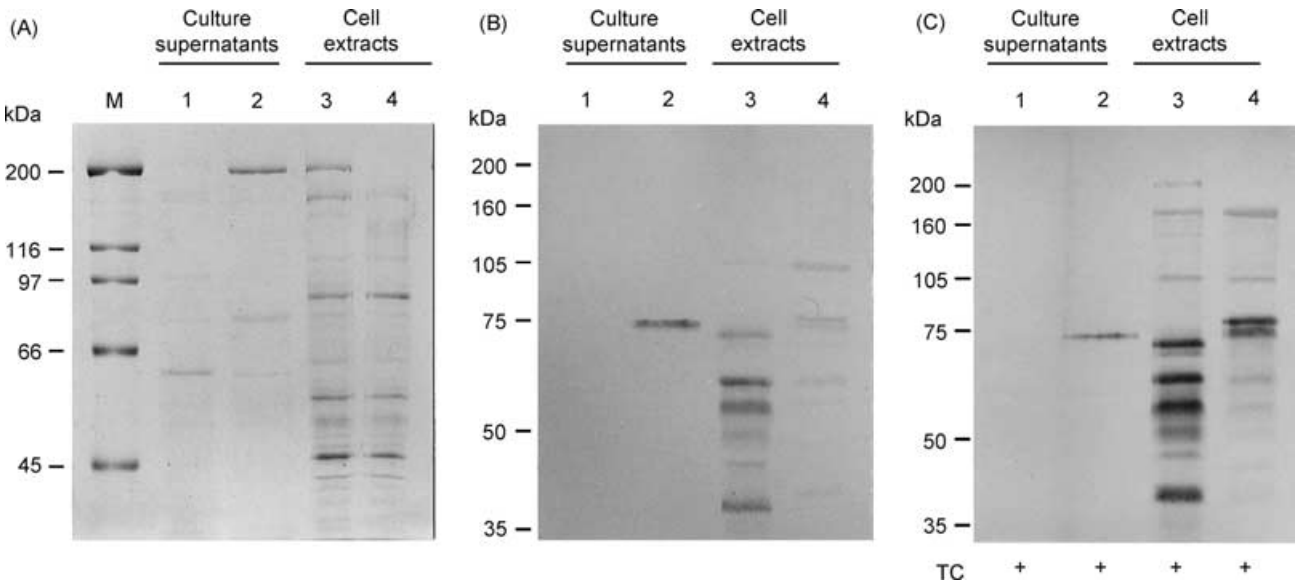

Fig. 1. Comparison of protein profiles between the wild type strain 109c and the *srtA*-deficient mutant of *S. mutans* 109c. Cell extracts and culture supernatants were prepared from *S. mutans* cells cultured in Todd Hewitt broth with (C) or without (A and B) 0.18 μg of tetracycline/ml and were subjected by SDS-PAGE. Equal amounts of proteins were added to each lane. A) Protein staining with Coomassie blue. (B and C) Western blot analysis with rabbit anti-GbpC serum. Lanes 1 and 3, wild type strain 109c; lanes 2 and 4, *srtA*-deficient mutant; lane M, size marker.

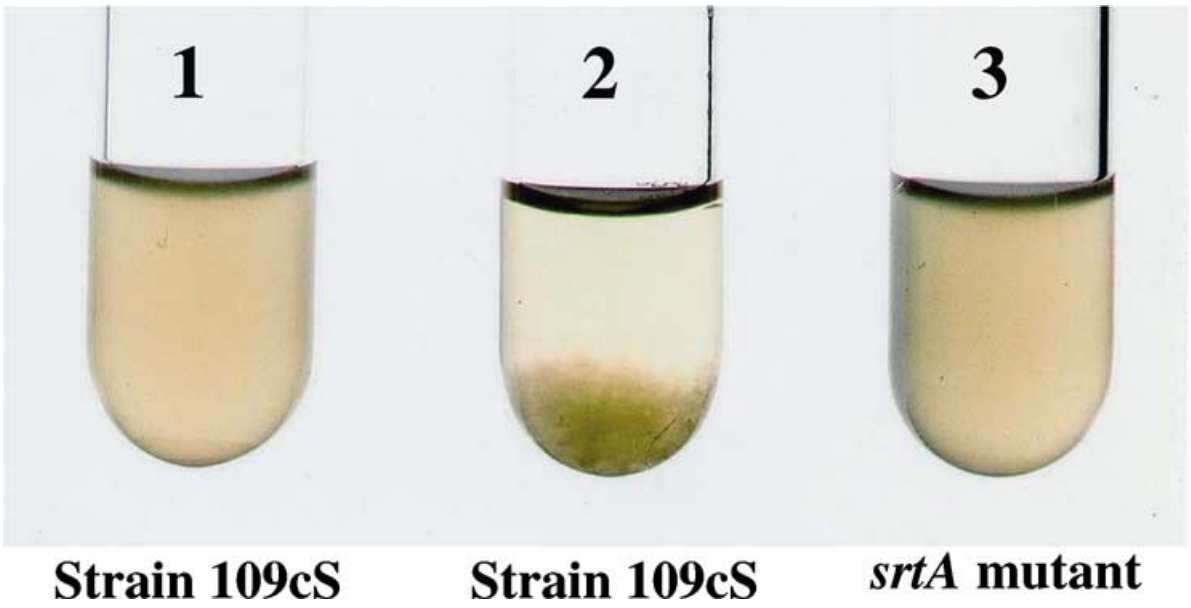

Fig. 2. Dextran-dependent aggregation of the wild type and the *srtA* mutant of *S. mutans* under a stress condition. *S. mutans* cells were grown overnight in BTR-G broth containing 0.18 μg of tetracycline/ml and then dextran T2000 was added into the tubes 2 and 3. Tubes 1 and 2, strain 109cS; tube 3, *srtA*-deficient mutant of strain 109cS.

shows the appearance of a dextran-dependent aggregation of the wild type and of the *srtA* mutant grown in BTR-G broth under a stress condition containing tetracycline. Aggregation of the *srtA* mutant cells was completely abolished even when incubation was prolonged up to 60 min, although the wild type cells strongly aggregated within a few minutes when dextran T2000 was added (Fig. 2, tubes 2 and 3), implying that GbpC anchored to the cell wall of *S. mutans* is essential for dextran-dependent aggregation of this organism. These results revealed that the 75 kDa protein released into the culture supernatant of the *srtA*-deficient mutant was GbpC, and that inactivation of the *srtA* gene in *S. mutans* inhibited cell wall-anchoring of GbpC mediated the dextran-dependent aggregation of *S. mutans* cells. Therefore, SrtA is involved in anchoring of GbpC to the cell wall in *S. mutans* and in the dextran-dependent aggregation by GbpC of this organism. Both strains 109c and 109cS showed the same results in Western blot analysis (Fig. 1) and dextran-dependent aggregation (Fig. 2).

S. mutans expresses several cell surface-associated proteins that mediate adherence to the tooth surfaces (5, 19), which is the first critical step in the development of dental caries. Several studies suggest that a surface protein termed PAc (also known as P1 and AgI/II) is necessary for adherence of *S. mutans* to saliva-coated hydroxyapatite (11, 22, 23) and that the anti-PAc antibody can prevent colonization of tooth surfaces by *S. mutans* (4, 10, 13). PAc has therefore been nominated as an effective immunogen against dental caries (10, 13, 22). *S. mutans* also expresses a surface protein GbpC that enables those cells to aggregate in the presence of dextran under stress conditions (Fig. 1B) (25, 27). It is suggested that GbpC is responsible for adherence of *S. mutans* to saliva-coated hydroxyapatite and may play an important role in the cariogenicity of *S. mutans* (18). Genetic studies have shown that PAc- and GbpC-deficient mutants of *S. mutans* cannot adhere to the tooth surfaces (1, 2, 12, 18). In the present and previous studies, inactivation of the *srtA* gene caused *S. mutans* to lose both PAc and GbpC on the cell surface (8) (Fig. 1B), implying that the *S. mutans* mutant lacking SrtA would lose physiologic functions mediated by PAc and GbpC molecules. These phenomena would be expected not only by inactivation of the *srtA* gene but also by inhibition of the SrtA enzyme of *S. mutans*. Therefore, *S. mutans* SrtA could be a more attractive target for the prevention of dental caries.

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References

1. Bowen WH, Schilling K, Giertsen E, et al. Role of a cell surface-associated protein in adherence and dental caries. Infect Immun 1991: **59**: 4606–4609.
2. Crowley PJ, Brady LJ, Michalek SM, Bleiweis AS. Virulence of a *spaP* mutant of *Streptococcus mutans* in a gnotobiotic rat model. Infect Immun 1999: **67**: 1201–1206.
3. Garandeau C, Reglier-Poupet H, Dubail I, Beretti JL, Berche P, Charbit A. The sortase SrtA of *Listeria monocytogenes* is involved in processing internalin and in virulence. Infect Immun 2002: **70**: 1382–1390.
4. Hajishengallis G, Nikolova E, Russell MW. Inhibition of *Streptococcus mutans* adherence to saliva-coated hydroxyapatite by human secretory immunoglobulin A (S-IgA) antibodies to cell surface protein antigen I/II: reversal by IgA1 protease cleavage. Infect Immun 1992: **60**: 5057–5064.
5. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980: **44**: 331–384.
6. Igarashi T, Yamamoto A, Goto N. Sequence analysis of the *Streptococcus mutans* Ingbritt *dexA* gene encoding extracellular dextranase. Microbiol Immunol 1995: **39**: 852–860.
7. Igarashi T, Morisaki H, Yamamoto A, Goto N. An essential amino acid residue for catalytic activity of the dextranase of *Streptococcus mutans*. Oral Microbiol Immunol 2002: **17**: 193–196.
8. Igarashi T, Asaga E, Goto N. The sortase of *Streptococcus mutans* mediates cell wall anchoring of a surface protein antigen. Oral Microbiol Immunol 2003: **18**: 266–269.
9. Jonsson IM, Mazmanian SK, Schneewind O, Verdrengh M, Bremell T, Tarkowski A. On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein

anchoring in murine septic arthritis. J Infect Dis 2002: **185**: 1417–1424.
10. Katz J, Harmon CC, Buckner GP, Richardson GJ, Russell MW, Michalek GJ. Protective salivary immunoglobulin A responses against *Streptococcus mutans* infection after intranasal immunization with *S. mutans* antigen I/II coupled to the B subunit of Cholera toxin. Infect Immun 1993: **61**: 1964–1971.
11. Koga T, Okahashi N, Takahashi I, Kanamoto T, Asakawa H, Iwaki M. Surface hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of *Streptococcus mutans* serotype c. Infect Immun 1990: **58**: 289–296.
12. Lee SF, Progulske-Fox A, Erdos GW, Piacentini DA, Ayakawa GY, Crowley PJ, et al. Construction and characterization of isogenic mutants of *Streptococcus mutans* deficient in major surface protein antigen P1 (I/II). Infect Immun 1989: **57**: 3306–3313.
13. Lehner T, Caldwell J, Smith R. Local passive immunization by monoclonal antibodies against Streptococcal antigen I/II in the prevention of dental caries. Infect Immun 1985: **50**: 796–799.
14. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986: **50**: 353–380.
15. Mazmanian SK, Liu G, Ton-That H, Schneewind O. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science 1999: **285**: 760–763.
16. Mazmanian SK, Liu G, Jensen ER, Lenoy E, Schneewind O. *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc Natl Acad Sci USA 2000: **97**: 5510–5515.
17. Murakami Y, Nakano Y, Yamashita Y, Koga T. Identification of a frameshift mutation resulting in premature termination and loss of cell wall anchoring of the PAc antigen of *Streptococcus mutans* GS-5. Infect Immun 1987: **65**: 794–797.
18. Nakano K, Matsumura M, Kawaguchi M, Fujiwara T, Sobue S, Nakagawa I, et al. Attenuation of glucan-binding protein C reduces the cariogenicity of *Streptococcus mutans*: analysis of strain isolated from human blood. J Dent Res 2002: **81**: 376–379.
19. Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 1999: **63**: 174–229.
20. Pallen MJ, Lam AC, Antonio M, Dunbar K. An embarrassment of sortases – a richness of substrates? Trends Microbiol 2001: **9**: 97–101.
21. Perry D, Kuramitsu HK. Genetic transformation of *Streptococcus mutans*. Infect Immun 1981: **32**: 1295–1297.
22. Russell MW. Immunization against dental caries. Curr Opin Dent 1992: **2**: 72–80.
23. Russell MW, Masson-Raherntulla B. Interaction between surface protein antigen of *Streptococcus mutans* and human salivary components. Oral Microbiol Immunol 1989: **4**: 106–111.
24. Sato Y, Yamamoto Y, Suzuki R, Kizaki H, Kuramitsu HK. Construction of *scrA*::*lacZ* gene fusions to investigate regulation of the sucrose PTS of *Streptococcus mutans*. FEMS Microbiol Lett 1991: **79**: 339–346.
25. Sato Y, Yamamoto Y, Kizaki H. Cloning and sequence analysis of the *gbpC* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. Infect Immun 1997: **65**: 668–675.
26. Sato Y, Yamamoto Y, Kizaki H. Xylitol-induced elevated expression of the *gbpC* gene in a population of *Streptococcus mutans* cells. Eur J Oral Sci 2000: **108**: 538–545.
27. Sato Y, Senpuku H, Okamoto K, Hanada N, Kizaki H. *Streptococcus mutans* binding to solid phase dextran mediated by the glucan-binding protein C. Oral Microbiol Immunol 2002: **17**: 252–256.
28. Ton-That H, Liu G, Mazmanian SK, Faull KF, Schneewind O. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. Proc Natl Acad Sci USA 1999: **96**: 12424–12429.