Oral Microbiology Immunology 2004: 19: 102–105
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology

Roles of *Streptococcus mutans* dextranase anchored to the cell wall by sortase

T. Igarashi, E. Asaga, N. Goto
Department of Oral Microbiology, Showa University School of Dentistry, Tokyo, Japan

Igarashi T, Asaga E, Goto N. Roles of Streptococcus mutans *dextranase anchored to the cell wall by sortase.*
Oral Microbiol Immunol 2004: 19: 102–105. © Blackwell Munksgaard, 2004.

In order to clarify the role that sortase (SrtA) plays in anchoring dextranase (Dex) to the cell wall of *Streptococcus mutans*, both Dex^- and $SrtA^-$ mutants were constructed by insertional inactivation of the respective genes. Western blot analysis with a Dex antiserum showed that in the *srtA* mutant the Dex was not bound to the cell wall but was secreted into the culture supernatant. In contrast, in the wild type, Dex remained cell-wall-associated. Biological properties of the *srtA* mutant were examined in dextran fermentation, colony morphology and adherence to a smooth surface. The *srtA* mutant, as well as the wild type, retained the ability to ferment dextran. However, the colony morphology of the *srtA* mutant on Todd Hewitt agar containing sucrose was much larger than that of the wild type and showed a ring-like structure. In addition, the *srtA* mutant was more adhesive to a smooth surface than the wild type when sucrose was present. However, the adhesion of the *srtA* mutant remarkably decreased by addition of exogenous dextranase. These studies suggest that the SrtA mediates Dex-anchoring to the cell wall in *S. mutans*, and cell wall-anchored Dex plays a role in controlling both the adhesive properties of extracellular glucan and the ability to utilize extracellular glucan as a nutrient source. In contrast, extracellular Dex is only responsible for degrading extracellular glucan as a nutrient source.

Key words: biofilm, dextranase; LPXTG motif; sortase; *Streptococcus mutans*

Takeshi Igarashi, Department of Oral Microbiology, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
Tel.: +81 3 3784 8166;
fax: +81 3 3784 8012;
e-mail: igatakes@dent.showa-u.ac.jp
Accepted for publication October 14, 2003

Dextranase (Dex) is an enzyme that hydrolyzes α-1,6-linkage in a glucan molecule produced from sucrose by mutans streptococci (4, 5). Oral streptococci, in particular *Streptococcus mutans*, are major producers of Dex (23, 25). Previous studies suggest that Dex is involved in the control of both the amount and the content of extracellular glucan and in the metabolic utilization of sugar (1, 25). Consequently, Dex is thought to be one of the virulence factors in *S. mutans*.

In recent studies it has been shown that surface proteins containing a C-terminal sorting signal with an LPXTG motif are covalently linked to the cell wall by a transpeptidase designated sortase (SrtA) that has been identified in many gram-positive bacteria (18, 20, 24). This suggests that the cell wall-anchored proteins are associated with the pathogenesis of gram-positive bacteria (3, 14, 16). In a more recent study we determined the complete nucleotide sequence of the *srtA* gene of *S. mutans* and demonstrated that SrtA catalyzes anchoring of a surface protein antigen (PAc) and glucan-binding protein C (GbpC) to the cell wall in *S. mutans* (12, 13).

The gene encoding Dex was cloned from *S. mutans* Ingbritt (6), and a subsequent sequence analysis showed that Dex is an LPXTG protein which possesses a C-terminal sorting signal (7). This structural feature strongly suggests that the *S. mutans* Dex is a cell wall-anchored enzyme protein, although Dex has previously been thought to be an extracellular enzyme (5, 25). However, the mechanism of cell wall anchoring of the Dex in *S. mutans* has not been clarified. In this study we examined cell wall anchoring of the *S. mutans* Dex by SrtA and the biological function of the cell wall-anchored Dex in *S. mutans*.

Materials and methods

Bacterial strains

S. mutans 109c was grown in Todd Hewitt broth (TH broth; Difco Laboratories, Detroit, MI) (12). *Escherichia coli* JM109 is routinely used as a plasmid host and grown in Luria-Bertani broth.

DNA extraction

Chromosomal DNA of *S. mutans* was purified by ultracentrifugation in a CsCl-ethidium bromide density gradient as described previously (7). Plasmid was extracted by a

Wizard miniprep purification kit (Promega, Madison, WI).

Construction of *S. mutans* mutants

A *srtA*-deficient mutant of *S. mutans* 109c was constructed previously by insertional inactivation of the *srtA* gene (12). A *dex*-deficient mutant of *S. mutans* 109c was also prepared by insertional inactivation of the *dex* gene. Briefly, an internal portion (411 bp) of the *dex* gene amplified with the primer pair Bam1131F-Eco1541R was cloned into a pUCE vector composed of a pUC19 vector with an erythromycin-resistant gene (12) and named pUE411dex. The nucleotide sequences of a pair of polymerase chain reaction (PCR) primers, Bam1131F and Eco1541R were as follows: Bam1131F, 5′-AAT TGG ATC CCG GTT TTG ATG GCT GGC AGG-3′ and Eco1541R, 5′-AAT TGA ATT CAA AAC GTC ACG CGC TGC ACC-3′. The recombinant plasmid pUE411dex was introduced into *S. mutans* 109c cells (21). Recombination between the internal portion (411 bp) and the homologous gene resulted in insertional inactivation of the *dex* gene. Transformants were selected on TH agar containing erythromycin. Insertion of the pUE411dex in the *dex* gene of *S. mutans* 109c was confirmed by PCR and Southern hybridization analyses (data not shown). PCR was performed under conditions described previously (10). In addition, loss of Dex activity of the *dex* mutant was verified by using a blue dextran agar plate (6, 8) and active staining with SDS-PAGE containing blue dextran (5, 6).

Western blotting

Whole cells were suspended in 1% sodium dodecyl sulfate-1% 2-mercaptoethanol, heated at 100°C for 5 min and centrifuged as reported previously (12, 17). The resulting supernatant was used as the cell extract. The cell extract and the culture supernatant were analyzed by Western blotting with rabbit anti-Dex serum as reported previously (11).

Colony morphology

Colony morphology of *S. mutans* 109c, the *srtA* mutant, and the *dex* mutant was observed on TH agar supplemented with 1% sucrose as described by Colby et al. (1). Colonies were allowed to grow anaerobically for 72 h at 37°C and plates were then transferred to 4°C. After 3 days at 4°C, differences of colony morphology between the wild type and the mutants were compared.

Adherence assay

The adherence of *S. mutans* cells was examined by modification of the method described by Larrimore et al. (15). *S. mutans* was grown overnight in TH broth containing 0.5% sucrose in a 24-well tissue culture plate. Exogenous Dex from *Penicillium* sp. (20 U; Sigma, St. Louis, MO) was added if required. After anaerobic incubation at 37°C overnight, the culture supernatant was gently removed and the plate was washed twice with water to remove non-adherent cells. Attached cells were stained with a crystal violet solution for 5 min and washed several times with water.

Dextran fermentation

The dextran fermentation of *S. mutans* strains was tested as described by Colby et al. (1). Briefly, cells from overnight TH cultures were washed twice with sterile saline and added to TH broth with or without 0.5% dextran T10. After overnight incubation at 37°C, the pH of the culture supernatant was measured.

Results

To examine whether Dex was anchored to the cell wall of *S. mutans* by SrtA, protein profiles in the culture supernatants and the cell extracts of the wild type 109c and the *srtA* mutant were compared. Protein staining with Coomassie blue did not detect the protein band corresponding to the expected molecular size (96 kDa) (5) of Dex in any fraction (Fig. 1A). However, Western blot analysis with anti-Dex serum showed Dex from the *srtA* mutant in the culture supernatant. The Dex from the wild type 109c was cell surface bound (Fig. 1B, lanes 2 and 3). The molecular sizes of the Dexs of the *srtA* mutant and the wild type were 102 kDa and 92 kDa, respectively. Several faint bands that were degradation products of the 92 kDa Dex were observed in the cell extract of the wild type (Fig. 1B, lane 3). In the cell extract of the *srtA* mutant, the 92 kDa band apparently disappeared (Fig. 1B, lane 4) and the 102 kDa band was not detected in the culture supernatant of the wild type 109c (Fig. 1B lane 1). In contrast, the 92 and 102 kDa bands were not detected in the *dex* mutant (data not shown).

Biological properties of the *srtA* mutant were investigated in dextran fermentation, colony morphology, and adherence to a smooth surface. In dextran fermentation, acid production from dextran was compared between the wild type 109c and the *srtA* mutant. The *dex* mutant was used as a control. When grown in TH broth containing dextran T10, both the *srtA* mutant and the wild type were able to ferment the dextran, but the *dex* mutant did not ferment dextran (Fig. 2). There were remarkable differences in the colonial morphology of the wild type and mutants when grown on sucrose agar. The *srtA* mutant colony was larger than the wild type colony, with a ring-like structure (Fig. 3A, B). In an adherence assay, the *srtA* mutant showed greater adherence to the surface of a plastic plate than the strain 109c when grown in the presence of sucrose (Fig. 4, wells 1 and 2).

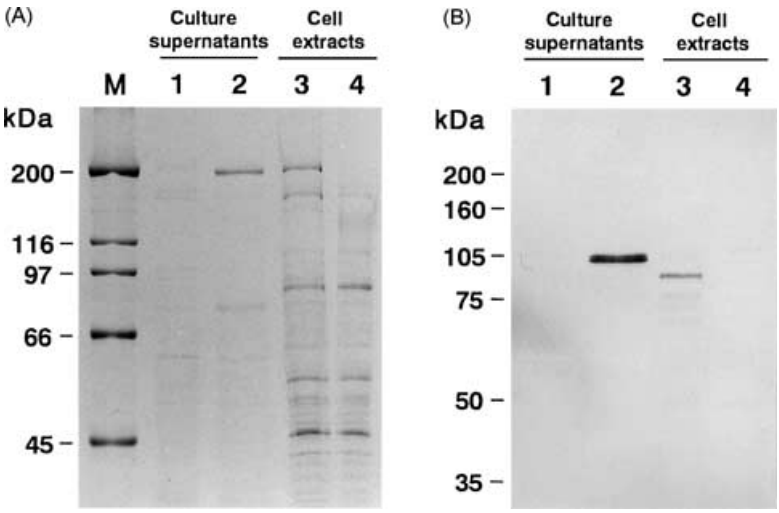

Fig. 1. Comparison of protein profiles between the wild type strain 109c and the *srtA*-deficient mutant of *S. mutans* 109c. Cell extracts and culture supernatants were prepared from *S. mutans* cells cultured in Todd Hewitt broth and were subjected by SDS-PAGE. (A) Protein staining with Coomassie blue. (B) Western blot analysis with rabbit anti-Dex serum. Lanes 1 and 3, wild type strain 109c; lanes 2 and 4, *srtA*-deficient mutant; lane M, size marker.

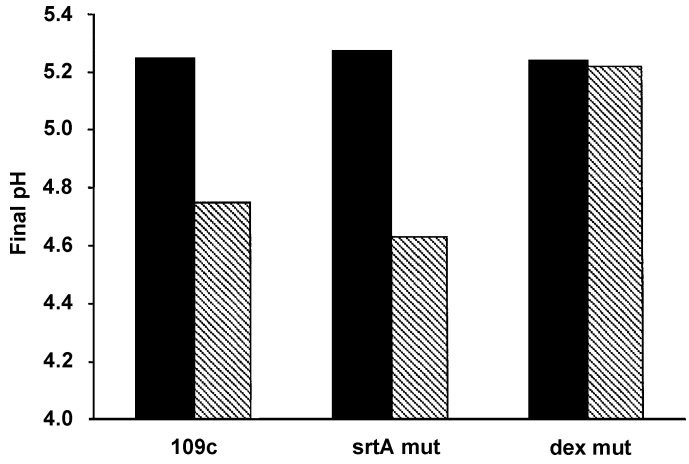

Fig. 2. Dextran fermentation of *S. mutans*. *S. mutans* 109c, *srtA* mutant, and *dex* mutant were grown in Todd Hewitt broth (solid bars) or Todd Hewitt broth with 0.5% dextran T10 (hashed bars). The pH of the culture was measured after 18 h.

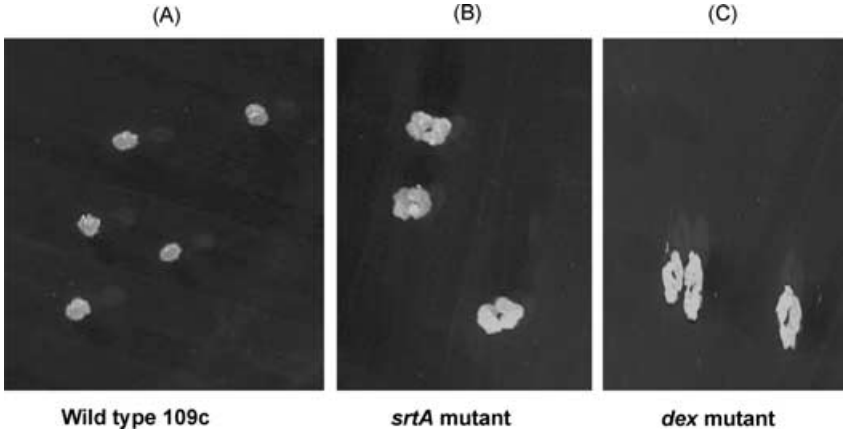

Fig. 3. Colony morphology of *S. mutans* 109c, the *srtA* mutant and the *dex* mutant. *S. mutans* cells were grown in a Todd Hewitt agar plate with 1% sucrose. Plates were incubated anaerobically at 37°C for 72 h and then aerobically at 4°C for 3 days. (A) wild type 109c, (B) *srtA* mutant, (C) *dex* mutant.

However, addition of exogenous dextranase from *Penicillium* sp. to the cultures resulted in a remarkable decrease of adherence level of the *srtA* mutant when compared with that of strain 109c (Fig. 4, wells 4 and 5). The colony morphology and the adherence assay of the *srtA* mutant were fundamentally similar with those of the *dex* mutant (Figs 3B, C and 4, wells 2 and 3, 5 and 6) (1).

Discussion

Insertional inactivation of the *srtA* gene resulted in loss of binding of the Dex to the cell surface and its appearance in the culture supernatant. This suggests that the *S. mutans* Dex is a cell surface protein linked to the cell wall by SrtA. In Western blot analysis, anti-Dex serum detected the proteins of 102 kDa in the supernatant of the *srtA* mutant and 92 kDa with smaller products in the wild type cell, respectively (Fig. 1B). The *dex* mutant failed to produce these immunoreactive proteins (data not shown), confirming that the proteins detected with anti-Dex serum were derived from the *dex* gene. The protein (102 kDa) in the culture supernatant of the *srtA* mutant was larger than the 92 kDa protein in the cell extract of the wild type. This is because the 102 kDa protein retains a C-terminal sorting signal with an LPXTG motif, as this protein is not processed by SrtA in the *srtA*-deficient mutant.

Genes encoding Dexs have been cloned from *S. mutans* (7), *Streptococcus sobrinus* (26), *Streptococcus downei* (10), *Streptococcus rattus* (9), *Streptococcus salivarius* (19) and *Streptococcus suis* (22), although only Dexs from mutans streptococci possess a C-terminal sorting signal (7, 10). This suggests that a cell wall-anchored form of Dex could play an important role in the pathogenesis of mutans streptococci. Both the *srtA* mutant and the wild type retained the ability to ferment dextran, indicating that both cell bound and extracellular dextranase contributed to the utilization of dextran in *S. mutans* (Fig. 2). In contrast, the *dex* mutant did not ferment this polysaccharide (Fig. 2). The morphology of colonies grown on a TH agar plate containing sucrose showed remarkable differences between the *srtA* mutant and the wild type. The colony of the *srtA* mutant was much larger than that of the wild type and showed a ring-like structure. These features were similar to the colony morphology of the *dex* mutant on TH-sucrose agar (Fig. 3)(1). Unlike the *dex* mutant, which is defective in Dex production, the *srtA* mutant produced Dex and released it into the culture supernatant. This suggests that the colony morphology of *S. mutans* on TH-sucrose agar is influenced not only by a deficiency of Dex but also by an alteration in the localization of Dex. The importance of surface-localized Dex was also supported by sucrose-dependent adherence of *S. mutans* cells. In the adherence assay, the *srtA* mutant adhered to the plastic plate to a greater extent than the wild type (Fig. 4). However, this increased adherence was markedly diminished by addition of exogenous dextranase. The same phenomenon was also observed in the adherence assay of the *dex* mutant (Fig. 4)(1). It is thought that the cariogenic potential of *S. mutans* is closely associated with the production of α-1, 3-rich, branched water-insoluble glucan, which is the major contributor to adherence to the tooth surface (2, 25). As Dex is an enzyme that hydrolyzes α-1,6-linkage in the glucan (5, 25), it is thought that this activity may modify glucan by altering the ratio of α-1,6- to α-1,3-linked chains, hence influencing solubility and adhesive properties (1, 25). Therefore, Dex would contribute to sucrose-dependent adherence by decreasing the proportion of α-1,6-linkage in the glucan. That the sucrose-dependent adherence of the *dex*- or *srtA*-deficient mutant, not the wild type 109c, of *S. mutans* was markedly diminished by exogenous dextranase (Fig. 4)(1) implies that the glucan produced by these mutants has more α-1,6-linkages than the wild type glucan. This result also indicates that the cell wall-anchored Dex, not extracellular Dex, is essential for the sucrose-dependent adherence. In this study we therefore propose that the *S. mutans* Dex anchored to the cell wall by SrtA is responsible both for modulating the solubility and the adhesive properties of extracellular glucan and for

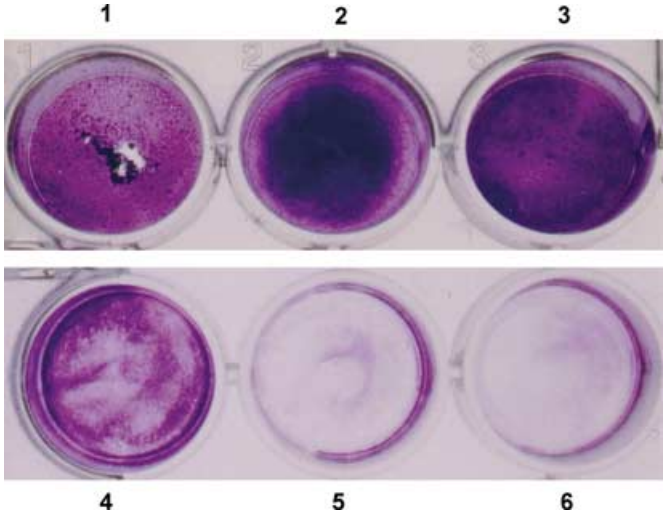

Fig. 4. Adherence of *S. mutans* cells on a plastic tissue culture plate. *S. mutans* 109c, the *srtA* mutant and the *dex* mutant were grown in Todd Hewitt broth-0.5% sucrose with (wells 4, 5 and 6) or without (wells 1, 2 and 3) exogenous Dex from *Penicillium* species. After overnight growth, unattached cells were removed by washing and adherent cells were stained with crystal violet. Wells 1 and 4, wild type 109c; wells 2 and 5, *srtA* mutant; wells 3 and 6, *dex* mutant.

utilizing extracellular glucan as a carbohydrate source. On the other hand, extracellular Dex is only associated with metabolic utilization of extracellular glucan.

The present and previous studies have shown that the *S. mutans* SrtA is involved in cell wall anchoring of Dex, PAc, and GbpC (this study, 12, 13), implying that the *S. mutans* mutant lacking SrtA loses biological functions mediated by Dex, PAc, and GbpC molecules on the cell surface of *S. mutans*. These phenomena appear to occur not only as the result of inactivation of the *srtA* gene but also due to the inhibition of the SrtA enzyme of *S. mutans*. SrtA of *S. mutans* could therefore be an attractive target for the prevention of biofilm formation and subsequent dental caries.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (no. 14571798 and no. 14571749) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Colby SM, Whiting GC, Tao L, Russell RRB. Insertional inactivation of the *Streptococcus mutans dexA* (dextranase) gene results in altered adherence and dextran catabolism. Microbiology 1995: **141**: 2929–2936.
2. Ebisu S, Misaki A, Kato K, Kotani S. The structure of water-insoluble glucans of cariogenic *Streptococcus mutans*, formed in the absence and presence of dextranase. Carbohydr Res 1974: **38**: 374–381.
3. Garandeau C, Reglier-Poupet H, Dubail I, et al. The sortase SrtA of *Listeria monocytogenes* is involved in processing internalin and in virulence. Infect Immun 2002: **70**: 1382–1390.
4. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980: **44**: 331–384.
5. Igarashi T, Yamamoto A, Goto N. Characterization of the dextranase purified from *Streptococcus mutans* Ingbritt. Microbiol Immunol 1992: **39**: 969–976.
6. Igarashi T, Yamamoto A, Goto N. Characterization of the dextranase gene (*dex*) of *Streptococcus mutans* and its recombinant product in an *Escherichia coli* host. Microbiol Immunol 1995: **39**: 387–391.
7. Igarashi T, Yamamoto A, Goto N. Sequence analysis of the *Streptococcus mutans* Ingbritt *dexA* gene encoding extracellular dextranase. Microbiol Immunol 1995: **39**: 852–860.
8. Igarashi T, Yamamoto A, Goto N. Detection of dextranase-producing gram-negative oral bacteria. Oral Microbiol Immunol 1998: **13**: 382–386.
9. Igarashi T, Yamamoto A, Goto N. Sequence analysis of the dextranase gene of *Streptococcus rattus*. J Dent Res 2000: **79** (Special issue): 224.
10. Igarashi T, Yamamoto A, Goto N. Nucleotide sequence and molecular characterization of a dextranase gene from *Streptococcus downei*. Microbiol Immunol 2001: **45**: 341–348.
11. 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.
12. 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.
13. Igarashi T, Asaga E, Sato Y, Goto N. Inactivation of *srtA* gene of *Streptococcus mutans* inhibits dextran-dependent aggregation by glucan-binding protein C. Oral Microbiol Immunol 2004: **19**: 57–60.
14. 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.
15. Larrimore S, Murchison H, Shiota T, Michalek SM, Curtiss RIII. *In vitro* and *in vivo* complementation of *Streptococcus mutans* mutants defective in adherence. Infect Immun 1993: **42**: 558–566.
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. 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.
19. Ohnishi Y, Kubo S, Ono Y, et al. Cloning and sequencing of the coding for dextranase from *Streptococcus salivarius*. Gene 1995: **156**: 93–96.
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. Serhir B, Dugourd D, Jacques M, Higgins R, Harel J. Cloning and characterization of a dextranase gene (*dexS*) from *Streptococcus suis*. Gene 1997: **190**: 257–261.
23. Staat RH, Schachtele CF. Evaluation of dextranase production by the cariogenic bacterium *Streptococcus mutans*. Infect Immun 1974: **9**: 467–469.
24. 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.
25. Walker GW, Pulkownik A, Morrey-Jones JG. Metabolism of the polysaccharides of human dental plaque: release of dextranase in batch cultures of *Streptococcus mutans*. J Gen Microbiol 1981: **127**: 201–208.
26. Wanda S-Y, Curtiss RIII. Purification and characterization of *Streptococcus sobrinus* dextranase produced in recombinant *Escherichia coli* and sequence analysis of the dextranase gene. J Bacteriol 1994: **176**: 3839–3850.