Role of Serotype-Specific Polysaccharide in the Resistance of *Streptococcus mutans* to Phagocytosis by Human Polymorphonuclear Leukocytes

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**Streptococcus mutans** has been strongly implicated as one of the causative organisms of dental caries. Phagocytosis by oral polymorphonuclear leukocytes (PMNs) is one of the most important mechanisms protecting against dental caries (31–34). Furthermore, *S. mutans* is associated with systemic diseases such as infectious endocarditis-associated glomerulonephritis and rheumatic fever, which can result when whole cells or components of *S. mutans* are translocated across the epithelial barrier and carried to target organs (36).

Several proteins involved in pathogenicity are located on the cell surface of *S. mutans* (12). A 190-kDa cell surface protein antigen (PAC) of *S. mutans* is one of the factors that mediate the binding of the organism to salivary components on tooth surfaces (11). The N-terminal region of this protein possesses an internal repeating amino acid sequence rich in alanine. The C-terminal region of PAC contains a potential transmembrane domain consisting primarily of hydrophobic amino acids, a cytoplasmic tail consisting of five charged amino acids, and a wall-spanning region rich in proline. These were inferred from analogy with streptococcal M protein, which is the major virulence factor of group A streptococci (6, 12). M protein is a fibrillar surface molecule that protects the bacteria from being ingested and killed by the host’s phagocytic cells (15, 28). This effect appears to result from the inhibition of phagocytosis and opsonization (41). Therefore, the *S. mutans* PAC protein antigen is thought to have anti-phagocytic activity, like the streptococcal M protein. However, we found no reports on the function of the *S. mutans* PAC protein in resistance to human phagocytes. In addition, glucosyltransferase I (GTF-I) and glucosyltransferase SI (GTF-S), which primarily produce water-insoluble glucan, a major cariogenic factor, are also observed on the cell surface of *S. mutans* (12, 13).

The serotype-specific polysaccharide antigens of *S. mutans* are cell wall polysaccharides that consist of a backbone structure of 1,2- and 1,3-linked rhamnosyl polymers with glucose side chains (19, 29, 42). *S. mutans* strains are classified into three serotypes, c, e, and f, whose serotype-specific polysaccharides have different linkages of glucose side chain (19, 29). In vitro stimulation of human monocytes with the serotype f-specific polysaccharide antigen induces the release of inflammatory cytokines, such as tumor necrosis factor alpha and interleukin-1β (35), and provokes nitric oxide production in the rat aorta (20). However, the function of the serotype-specific polysaccharide antigens in phagocytosis of the organism by human phagocytes has not been reported.

We constructed various mutant strains of *S. mutans* defective in cell-surface components by using insertional inactivated mutagenesis. In this study, we examined the chemiluminescence (CL) responses of human PMNs to these mutants to investigate the role of the cell surface components in the interaction between *S. mutans* and PMNs. Furthermore, the function of the polysaccharide was examined by transmission electron microscope. In this report, we discuss the function of the serotype-specific polysaccharide of *S. mutans* in resistance to phagocytosis by human phagocytes.
Preparation of human PMNs. Human PMNs were prepared from peripheral blood obtained from three healthy volunteers. PMNs were isolated with Mono-Poly resolving medium (Dainippon Pharmaceutical Co., Tokyo, Japan). 

MATERIALS AND METHODS

Bacterial strains. The S. mutans strains used in this study are listed in Table 1. S. mutans wild-type strains Xc (serotype c), LM7 (serotype c), KT6219 (serotype f), Xc11 (PAc-defective mutant), and Xc24R (serotype c-specific polysaccharide-defective mutant) were selected from the stock culture collection in the Department of Preventive Dentistry, Kyushu University Faculty of Dentistry, Fukuoka, Japan. Strains of S. mutans were grown at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). For transformants of S. mutans, erythromycin at a final concentration of 10 μg/ml was added.

DNA manipulation. Preparation of chromosomal DNA from the S. mutans rmlB mutant strain Xc24R and transformation of S. mutans strains LM7 and KT6219 were carried out as described previously (27). Transformants were isolated by selecting colonies containing 10 μg of erythromycin per ml.

Immunoblotting. S. mutans strains were grown to the stationary phase in 20 ml of BHI broth. The bacterial cells were harvested, suspended in 200 μl of 10 mM Tris-HCl buffer (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 1% mercaptoethanol, and 20% glycerol, and heated at 100°C for 5 min. The cell extracts were then clarified by centrifugation. Seven-microliter samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE [7.5% polyacrylamide]) and electrophoretically transferred to a nitrocellulose membrane (3, 14). After being blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS [pH 7.4]) plus 0.1% Tween 20, the antibodies bound to proteins immobilized on the membranes were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Zymed Laboratories, South San Francisco, Calif.) and developed by using the addition of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt as the detection mixtures containing human PMNs (106 cells) were added to 20 ml of the bacterial suspension 10-fold diluted in RPMI 1640. The mixtures were incubated at 37°C for 90 min with gentle rotation. After incubation, the unengulfed bacteria were washed away with HBSS− buffer, and the washed human PMNs were fixed in 2.5% glutaraldehyde−2% paraformaldehyde at pH 7.4 and postfixed in 1% OsO4. After dehydration, these cells were embedded in Epon, and ultrathin sections were used for transmission electron microscopy with a JEM-1200EXII electron microscope (Japan Electron Optics Laboratories Co., Tokyo, Japan).

To examine the bacterial cell surface architecture, Xc and Xc24R cells were washed with 0.1 M sodium phosphate buffer (pH 7.4). The cells were then fixed, stained, and dehydrated under the same conditions as described above. After dehydration, cells were embedded in Epon, ultrathin sectioned, and used for transmission electron microscopy.

Hydrophobicity. The surface hydrophobicity of S. mutans was examined as described by Koga et al. (11). S. mutans strains were grown at 37°C for 18 h in BHI broth. Bacterial cells were washed and suspended in PUM buffer (11) to an OD600 of 0.6. Three hundred microliters of hexadecane was added to 3-ml samples of the cell suspension, mixed with a vortex mixer for 15 s, and allowed to stand until the phases separated. The OD of the lower aqueous phase was measured. The percentage loss in OD relative to the initial cell suspension, which is due to adsorption of S. mutans strains to hexadecane, was defined to be cell surface hydrophobicity of bacteria.

RESULTS

Construction of mutant strains of S. mutans. We previously reported that four rml genes (rmlA, rmlB, rmlC, and rmlD) are involved in dTDP-rhamnose synthesis from glucose 1-phosphate and that dTDP-rhamnose is used as an immediate precursor for the synthesis of the backbone of the serotype-specific polysaccharides of S. mutans (38, 39). Recently, we found that an rmlB-inactivated mutant (Xc24R) isolated on mitis salivarius agar exhibited the gene fusion between gtfB and gfcF, which encode GTF-I and GTF-SI, respectively. On the other hand, an rmlB-inactivated mutant which possesses intact gtfB and gfcF (Xc24R) was selected on tryptic soy agar in the absence of sucrose (46). We confirmed that the insertional inactivation of the rmlB gene did not affect the production of PAc, GTF-I, and GTF-SI in Xc24R (Fig. 1). We used Xc24R but not Xc24 as the serotype c-specific polysaccharide-defective mutant, because the surface architecture of Xc24R was consid-

TABLE 1. S. mutans strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Xc</td>
<td>Serotype c wild-type strain</td>
<td>10</td>
</tr>
<tr>
<td>Xc11</td>
<td>Em′, transformant of Xc; pac; PAc</td>
<td>47</td>
</tr>
<tr>
<td>Xc16</td>
<td>Em′, transformant of Xc; gtfB and gfcF; GTF-I' and GTF-SI'</td>
<td>This study</td>
</tr>
<tr>
<td>Xc24</td>
<td>Em′, transformant of Xc; rmlB, fusion between gtfB and gfcF; serotype c polysaccharide-</td>
<td>39</td>
</tr>
<tr>
<td>Xc24R</td>
<td>Em′, transformant of Xc; rmlB, intact gtfB and gfcF; serotype c polysaccharide-</td>
<td>46</td>
</tr>
<tr>
<td>LM7</td>
<td>Serotype c wild-type strain</td>
<td>21</td>
</tr>
<tr>
<td>LM7DR</td>
<td>Em′, transformant of LM7; rmlB; serotype c polysaccharide</td>
<td>This study</td>
</tr>
<tr>
<td>KT6219</td>
<td>Serotype f wild-type strain</td>
<td>This study</td>
</tr>
<tr>
<td>KT6219DR</td>
<td>Em′, transformant of KT6219; rmlB; serotype f polysaccharide</td>
<td>This study</td>
</tr>
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</table>

* Em′, erythromycin resistance.
ered to be less influenced than that of Xc24 by inactivation of the rmlB gene. In the present study, rmlB-inactivated transformants of LM7 (serotype e) and KT6219 (serotype f) were also isolated on tryptic soy agar by the same procedure used for Xc24R construction. These transformants were designated LM7DR and KT6219DR, as shown in Table 1. Formamide extracts of these two mutants did not react with any serotype-specific antisera in immunodiffusion (Fig. 2). Southern blot analysis revealed that the gtfB and gtfC genes in both two mutants were intact, and normal expression of these two genes was confirmed by Western blot analysis (data not shown). Strain Xc16, in which both gtfB and gtfC are inactivated with a 2.0-kb cat cartridge (47), was constructed by the same principles as described previously (45). The mutation was confirmed following Southern blot analysis of the chromosomal DNA of the mutant Xc16 (data not shown).

**Immunoblotting analysis.** Immunoblotting analysis showed that although anti-PAc serum detected the band of 190-kDa cell surface protein antigen in the extracts from strains Xc, Xc16, and Xc24R (Fig. 1A, lanes 1, 3, and 4, respectively), the band reacting with anti-PAc serum was not observed in Xc11 (Fig. 1A, lane 2). On the other hand, anti-GTF-I serum detected two prominent bands in the extracts from strains Xc, Xc11, and Xc24R (Fig. 1B, lanes 1, 2, and 4), because of extensive amino acid homology between GTF-I and GTF-SI enzymes (37). Xc16 did not have these two prominent bands but exhibited a smaller band at ~90 kDa (Fig. 1B, lane 3). The band at ~90 kDa in Xc16 may be derived from the truncated gene product of the gtfB gene, because half of the 5'-terminus portion of gtfB is still able to be translated in Xc16. Active staining analysis on SDS-PAGE revealed that Xc16 did not exhibit water-insoluble glucan-producing activity (data not shown). These results confirmed that strain Xc11 was a PAc-defective mutant and strain Xc16 was a mutant defective in both GTF-I and GTF-SI.

**CL responses of human PMNs to S. mutans strains.** The luminol-enhanced CL responses of human PMNs in the presence or absence of complement were examined when challenged with S. mutans Xc or its transformants. Figure 3 shows typical patterns of the CL response of human PMNs to S. mutans strains in the presence of complement. The CL response of human PMNs to the PAc-defective mutant Xc11 was as weak as it was for wild-type strain Xc. The response to mutant Xc16, which is defective in both GTF-I and GTF-SI, was also very weak. In contrast, the CL response of human PMNs to strain Xc24R, which is defective in serotype c-specific polysaccharide, was significantly stronger than the responses to

![FIG. 1. Western immunoblot detection of PAc, GTF-I, or GTF-SI expression in S. mutans strains. Surface proteins were extracted from the cell in the stationary phase of growth. The cell extracts were subjected to SDS-PAGE (7.5% polyacrylamide), proteins were blotted onto nitrocellulose membranes, and blots were probed with rabbit serum raised against recombinant PAc protein (A) or GTF-I (B) of S. mutans. Lanes: 1, strain Xc; 2, strain Xc11; 3, strain Xc16; 4, strain Xc24R.](http://iai.asm.org/)

![FIG. 2. Immunodiffusion of formamide extracts of S. mutans strains against rabbit antisera to whole cells of S. mutans strains MT8148 (serotype c) (A), LM7 (serotype e) (B), and OMZ175 (serotype f) (C). The outer wells contain formamide extracts from strains Xc (well 1), Xc11 (well 2), Xc16 (well 3), Xc24R (well 4), LM7 (well 5), LM7DR (well 6), KT6219 (well 7), and KT6219DR (well 8).](http://iai.asm.org/)

![FIG. 3. Representative CL responses of human PMNs to S. mutans strains in the presence of complement. Human PMNs (10⁶ cells) pretreated with HBSS⁺⁺ buffer containing 1% gelatin in the presence of 1% complement were unstimulated (○) or stimulated at 37°C for 90 min with whole cells of S. mutans strain (6.4 × 10⁶ CFU) Xc (●), Xc11 (▲), Xc16 (●), or Xc24R (■). The experiments were performed three times, and similar results were obtained in each experiment.](http://iai.asm.org/)
The cell-surface hydrophobicity of \( S. \) mutans strains was examined (Table 2). The surface hydrophobicity of strain Xc11 was significantly lower than that of wild-type strain Xc (\( P < 0.01 \)), while strain Xc24R was significantly more hydrophobic than strain Xc (\( P < 0.01 \)). The surface hydrophobicity of strain Xc16 was almost the same as that of strain Xc. Serotype-specific polysaccharide-defective mutants LM7DR and KT6219DR were also significantly more hydrophobic than parental strains LM7 (serotype e) and KT6219 (serotype f), respectively (\( P < 0.01 \)).

**Differences in cell surface architecture between strains Xc and Xc24R.** Electron microscopic observation of thin-sectioned cells of Xc and Xc24R indicated that there was a cell wall-like architecture which had two layers on the cell surfaces of both strain Xc and strain Xc24R (Fig. 6). In strain Xc, the outer layer which seemed to contain several cell surface components was wide and rather transparent, and the inner one, which was thought to be a peptidoglycan layer, showed a high density. Both of these layers in strain Xc24R were thinner than those in strain Xc, and Xc24R exhibited a dim contour of the outer layer.

**DISCUSSION**

Phagocytosis of bacterial pathogens by phagocytes, such as neutrophil leukocytes and macrophages, is one of the most important defense mechanisms in the oral cavity (2, 31, 32, 34). This function may regulate \( S. \) mutans in crevicular fluid and prevent dental caries, particularly root caries. There is evidence that patients with neutropenia are more susceptible to root caries (26), suggesting an important role of phagocytic cells in the host resistance to dental caries. Therefore, it is very important to study phagocytosis of \( S. \) mutans by human PMNs.

The luminol-enhanced CL method, which reflects phagocytic oxidative metabolism in PMNs, is often used for assessing phagocytosis (9). In this study, we examined the roles of cell surface components of \( S. \) mutans in resistance to phagocytosis by human PMNs by using a CL assay. The CL responses of human PMNs to parental strain Xc, PAc-defective mutant Xc11, and mutant Xc16, which is defective in both GTF-I and GTF-SI, were very weak (Fig. 3). The protein PAc is structurally similar to the M protein (6, 12). Therefore, it would be reasonable to assume that this protein may have antiphagocytic activity, like the M protein. However, the results of the CL assay and killing assay were not consistent with this hypothesis. In contrast, strain Xc24R defective in serotype c-specific polysaccharide induced a markedly strong CL response (Fig. 3). In addition, strain Xc24R was more efficiently killed by human PMNs than strains Xc, Xc11, and Xc16 (Fig. 4). Under light microscopy, many human PMNs were observed to form clusters when Xc24R were incubated with Xc24R (Fig. 5A), whereas few clustered human PMNs were observed when challenged with wild-type strain Xc (Fig. 5B). Observations by transmission electron microscopy showed that some human PMNs made clusters, and most of them internalized many Xc24R cells. On the other hand, few human PMNs internalized Xc cells (Fig. 5D).
GTF-SI protein were lost. Although we could not rule out a role of the GTF-I N terminus in antiphagocytic effect against PMNs, our findings suggest that GTF proteins may not play an important role in antiphagocytic effect.

Bacterial hydrophobicity is known to play an important role in the interaction between bacteria and phagocytic cells and subsequent phagocytosis (1, 7, 24, 40, 43). In this study, the cell surface of the serotype c-specific polysaccharide-defective mutant Xc24R is markedly more hydrophobic than that of the wild-type strain Xc (Table 2). This was consistent with the results of the CL assay (Fig. 3). The cell surface hydrophobicity of Xc16 (defective in both GTF-I and GTF-SI) was the same as

FIG. 5. Light (A and B) and transmission electron (C and D) microscopic observations of human PMNs incubated with S. mutans strain Xc24R (A and C) or strain Xc (B and D) in the presence of 2% complement for 90 min. Magnifications, ×80 (A and B) and ×2,500 (C and D).
Each value represents the mean ± standard deviation for assays performed five times. That of strain Xc, while that of the PAc-defective mutant Xc11 was much lower. These results do not completely match those of the CL assay. The CL response of human PMNs to strain Xc was the same as the background response in the absence of bacteria (Fig. 3). Moreover, the degrees of killing of Xc and Xc11 by human PMNs were very low, and the difference between both data was not significant (Fig. 4). These findings suggest that even wild-type strain Xc is rarely ingested by human PMNs. In the assays used in this study, it is difficult to show that Xc11 is more resistant to human phagocytic cells than Xc. Therefore, the results of the CL assay and the killing assay may not contradict those related to the cell surface hydrophobicity of S. mutans strains.

The electron microscopic observation of cell surfaces of S. mutans strains Xc and Xc24R indicated that the cell wall of Xc24R was thinner than that of parental strain Xc (Fig. 6). This difference is considered to be derived from the defectiveness of serotype-specific polysaccharide. Gram-positive bacteria such as S. mutans have a thick cell wall which is mainly composed of peptidoglycan and lipoteichoic acid (LTA). It was reported that LTA is the major factor responsible for cell surface hydrophobicity of group A streptococci and that LTA specifically binds to human PMNs (5, 22). The markedly high cell surface hydrophobicity of Xc24R may be due to the exposure of LTA on the cell surface. Furthermore, the exposure of LTA on the cell surface may elicit binding of Xc24R to human PMNs and subsequent phagocytosis.

The serum opsonic activity for S. mutans was reported to be closely related to protection against dental caries (34). Scully et al. (33) suggested that the opsonized S. mutans may fail to proliferate and may be phagocytosed and killed by local neutrophils and complement. Lehner et al. (16–18) also suggested that local passive immunization by monoclonal antibody may be a strong weapon for preventing dental caries. Although serotype-specific polysaccharide antigens of mutants streptococci were reported to be poorly immunogenic, oral administration of serotype c polysaccharide encapsulated in liposomes to human volunteers was reported to induce serum IgG and salivary IgA antibodies (30). This finding suggests that immunization with serotype-specific polysaccharide may prevent dental caries.

In conclusion, this study suggests that the serotype-specific polysaccharide antigens of S. mutans play an important role in resistance to phagocytosis and consequent killing by human PMNs. The serotype-specific polysaccharide antigens of S. mutans may make the cell surface more hydrophilic, which interferes with the interaction between this organism and the host phagocytic cells. It is speculated that inhibition of the rmlB gene product may be an effective method of controlling S. mutans. Antibodies against serotype-specific polysaccharide antigens of S. mutans may also be useful for controlling the organism and preventing dental caries.

ACKNOWLEDGMENTS

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REFERENCES


### Table 2. Surface hydrophobicity of S. mutans strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hydrophobicity (%)</th>
</tr>
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<tbody>
<tr>
<td>Xc</td>
<td>53.2 ± 2.2</td>
</tr>
<tr>
<td>Xc11</td>
<td>32.6 ± 2.3</td>
</tr>
<tr>
<td>Xc16</td>
<td>51.5 ± 3.5</td>
</tr>
<tr>
<td>Xc24R</td>
<td>99.3 ± 0.6</td>
</tr>
<tr>
<td>LM7</td>
<td>44.2 ± 3.5</td>
</tr>
<tr>
<td>LM7DR</td>
<td>78.6 ± 2.6</td>
</tr>
<tr>
<td>KT6219</td>
<td>49.9 ± 5.1</td>
</tr>
<tr>
<td>KT6219DR</td>
<td>82.7 ± 1.0</td>
</tr>
</tbody>
</table>

* The bacterial cell suspension (3 ml, OD<sub>550</sub> = 0.6) was mixed with 0.3 ml of hexadecane. The cell surface hydrophobicity was calculated as the percentage loss of OD of the aqueous phase relative to the initial bacterial cell suspension.

* P < 0.01 compared with the value of the wild-type strain of each serotype.