Effect of Subculturing on Expression of a Cell-surface Protein Antigen by *Streptococcus mutans*

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Two freshly isolated strains, Xc and Yc, of *Streptococcus mutans* serotype c from human dental plaque were subcultured 100 times in Brain Heart Infusion broth. The cell-surface hydrophobicity of strain Xc markedly decreased after subculturing 60 times, but that of strain Yc remained unaltered. Radioimmunoassay showed a close correlation between surface hydrophobicity and the amount of a cell-surface protein antigen (PAC) of *M*, 190000. One hydrophilic variant (strain Xc100L), one relatively hydrophobic variant (strain Xc100H), and two hydrophobic variants (strains Yc100H1 and Yc100H2) were isolated from the 100-fold subcultures of hydrophobic strains Xc and Yc, respectively. SDS-PAGE showed that the amount of cell-associated and cell-free PAC of strain Xc100L was smaller than that of strains Xc and Xc100H. Strain Yc100H2 produced larger amounts of cell-associated PAC than strains Yc and Yc100H1. Resting cells of hydrophilic strain Xc100L attached in smaller numbers to saliva-coated hydroxyapatite than did other hydrophobic strains. RNA dot-blot analysis demonstrated a significant decrease in PAC-specific mRNA in strain Xc100L, as compared with strains Xc and Xc100H. Neither rearrangement nor deletion in the structural gene (pac) for PAC of these strains was observed by Southern blot analysis. These findings suggest that a mechanism which regulates the transcription of the pac gene participates in the quantitative variation of PAC after repeated subculturing.

INTRODUCTION

*Streptococcus mutans* possesses a cell-free and wall-associated protein antigen of *M*, 190000 which has been variously designated as antigen B (Russell, 1979), I/II (Russell et al., 1980), IF (Hughes et al., 1980), P1 (Forester et al., 1983) and PAC (Okahashi et al., 1989a). *Streptococcus sobrinus* produces a protein antigen of *M*, 210000 named SpaA (Holt et al., 1982) or PAg (Okahashi et al., 1986), which shows serological cross-reactivity with the protein antigen of *S. mutans*. The wall-associated protein antigen of *S. mutans* is considered to be involved in forming hydrophobic bonds between the organism and hydrophobic regions of salivary pellicle on tooth surfaces (McBride et al., 1984; Knox et al., 1986; Okahashi et al., 1989a; Ohta et al., 1989).

It was reported that the cell-surface hydrophobicity of fresh isolates of *S. mutans* from human dental plaque decreased after repeated subculturing *in vitro* on blood agar (Westergren & Olsson, 1983). McBride et al. (1984) and Russell & Smith (1986) demonstrated that in fresh hydrophobic isolates the high-*M*, protein antigen is predominantly cell-associated, whereas in subcultured hydrophilic variants the antigen is free in culture supernatants. Little is known, however, of the mechanism of changes in the organization of the protein antigen after repeated subculturing.

In the present study, we subcultured two fresh isolates of *S. mutans* serotype c 100 times. We compared the subcultured variants with their parent strains in their cell-surface hydrophobicity.

Abbreviations: PAC, an *M*, 190000 protein antigen of *S. mutans* serotype c; S-HA, saliva-coated hydroxyapatite.
METHODS

Bacteria. S. mutans strains Xc and Yc were isolated from the dental plaque of two healthy men. Swab samples, collected with a cotton applicator from teeth, were serially diluted in 0.15 M NaCl and plated on sucrose/bacitracin-supplemented mitis-salivarius (MS-SB) agar (Gold et al., 1973). The MS-SB plates were incubated at 37 °C for 48 h. One colony from each individual (strains Xc and Yc) was purified by being streaked twice on the MS-SB agar, and cultured in 5 ml Brain Heart Infusion (BHI) broth (Difco) at 37 °C for 16 h. Biochemical tests and serotyping of these isolates were performed as described by Hamada et al. (1979) and Masuda et al. (1979), respectively. The original culture (0.1 ml) was transferred to 5 ml BHI broth and incubated at 37 °C for 48 h. Subculturing for 48 h was repeated 100 times, and all subcultures were lyophilized. Two variants were isolated from the 100-fold subculture of each fresh isolate.

Hydrophobicity. S. mutans strains were grown at 37 °C for 18 h in BHI broth. The cells were washed twice and suspended in PUM buffer (Rosenberg et al., 1980) to an OD550 of 0.6. Triplicate samples (3 ml) of the bacterial suspensions were placed in test tubes and 0.3 ml hexadecane was added. The tubes were then mixed with a vortex mixer for 1 min and allowed to stand until the phases separated. The OD550 of the lower aqueous phase was measured. Adsorption was calculated as the percentage loss in optical density relative to that of the initial cell suspension.

Adsorption of resting cells to saliva-coated hydroxyapatite. Spheroidal hydroxyapatite beads (20 mg) (BDH) were incubated with 1 ml clarified whole saliva for 1 h at room temperature, and washed three times with buffered KC1 (Eifert et al., 1984). S. mutans strains were grown at 37 °C for 18 h in BHI broth containing [methyl-3H]thymidine (62 Ci mmol⁻¹, 2.3 TBq mmol⁻¹; ICN Radiochemicals) at a final concentration of 10 μCi ml⁻¹. [3H]Thymidine-labelled S. mutans (5 × 10⁷ cells) was allowed to react with the saliva-coated hydroxyapatite (S-HA) beads in 1 ml buffered KC1 solution. After shaking at 37 °C for 1 h, the beads were washed twice with buffered KC1 solution. The radioactivity associated with the beads was then determined as described by Koga et al. (1986) and the number of bacteria adsorbed was determined from the calculated specific radioactivity of the bacteria.

Antiserum. Rabbit anti-PAC serum was prepared as previously described (Ohta et al., 1989). PAC is immunologically identical to antigen B kindly supplied by R. R. B. Russell, Royal College of Surgeons, Downe, UK (Russell, 1979; Okahashi et al., 1986).

Radioimmunoassay of PAC. S. mutans strains were grown at 37 °C for 18 h in BHI broth. The cells were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS, pH 7.5). The washed cells (5 × 10⁷) were allowed to react with rabbit anti-PAC serum (1 μl) in 150 μl PBS containing 1 % (w/v) bovine serum albumin for 1 h at room temperature. After washing three times in PBS by centrifugation, the cells were allowed to react with 1 μl 125I-labelled goat anti-rabbit immunoglobulin G (ICN Radiochemicals) in 50 μl PBS containing 1 % (w/v) bovine serum albumin. After incubation for 1 h at room temperature, the cells were washed three times in PBS. The radioactivity associated with the cells was then determined (Ohta et al., 1989).

SDS-PAGE. S. mutans strains were grown at 37 °C for 18 h in diffusate medium of BHI broth. The cell-free supernatant was collected by centrifugation. Extracellular proteins were precipitated from the culture supernatant by adding solid ammonium sulphate to 60 % saturation. The precipitate was collected by centrifugation, dissolved in distilled water to 1/100 of the original volume, dialysed extensively against distilled water, and lyophilized. The dried culture supernatant, and whole cells (50 mg dry weight ml⁻¹) were suspended separately in 10 mM-Tris/HCl, pH 6-8, containing 8 M-urea, 1 % (w/v) SDS and 1 % (v/v) 2-mercaptoethanol, and heated at 100 °C for 3 min. The cell extract was then clarified by centrifugation. The culture supernatant (0-5 mg protein) and the cell extract (50 μl) were subjected to SDS-PAGE on a 7.5 % (w/v) resolving and a 3 % (w/v) stacking slab gel containing 0-1 % (w/v) SDS (Laemmli, 1970).

Southern hybridization. The chimeric plasmid (pPC41) containing the structural gene (pac) encoding PAC (Okahashi et al., 1989a) was digested with restriction endonuclease PstI. PstI fragments of the pac gene were radio-labelled by nick translation (Maniatis et al., 1982) using [α-32P]dCTP (ICN Radiochemicals). Chromosomal DNAs from S. mutans strains were digested with BamHI, EcoRI, HindIII or PstI. The DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes (Maniatis et al., 1982). Hybridization on nitrocellulose membranes was performed with 50 % (v/v) formamide at 42 °C according to the method of Southern (1975).

RNA dot-blotting. S. mutans strains were grown in 100 ml Todd Hewitt broth (Difco) supplemented with 20 mM DL-threonine to a concentration of approximately 10⁸ cells ml⁻¹. The cells were harvested by centrifugation and
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suspended in 10 ml BHI broth supplemented with 30% (w/v) raffinose. The cell suspensions were incubated with 2 mg lysozyme ml⁻¹ for 30 min at 37 °C, and then with 0-1 mg N-acetylmuramidase ml⁻¹ (Seikagaku Kogyo) for 30 min at 37 °C. After incubation, the cells were harvested by centrifugation, suspended in 3 ml 4 M-guanidine thiocyanate containing 0-05% (w/v) N-lauroylsarcosine sodium salt, 0-1% (v/v) 2-mercaptoethanol and 25 mM-sodium citrate, and disrupted by drawing the suspensions through an 18-gauge needle. The disrupted cell suspensions were clarified by centrifugation, and CsCl (1-2 g each) was added. The solutions were then layered onto 1-4 ml 5-7 M-CsCl containing 0-1 M-EDTA, pH 7-0, in a polycollamer tube (Beckman), and centrifuged at 30000 r.p.m. for 16 h in an SW50.1 rotor (Beckman). The pelleted RNA was dissolved in 0-4 ml 0-3 M-sodium acetate, pH 7-0, and precipitated with 2-5 vols ethanol. The RNA preparations were washed with 70% (v/v) ethanol, and stored in distilled water at −70 °C until use.

RNA dot-blotting was performed as described by Thomas (1983). Briefly, the RNA preparations (20 μg each) were incubated at 50 °C for 1 h in 0-4 ml 10 mM-sodium phosphate buffer, pH 7-0, containing 1 mM-glyoxal. Serial twofold dilutions of the mixture were made in distilled water. RNA samples (0-2 ml each) were placed into wells of a Bio-Dot microfiltration apparatus (Bio-Rad) with a sheet of nitrocellulose membrane equilibrated with 3 M-NaCl containing 0-3 M-Na-citrate. The blot was dried, baked for 2 h at 80 °C, treated with 20 mM-Tris/HCl, pH 8-0, for 5 min at 100 °C, and prehybridized and hybridized as described by Thomas (1983). The 1-5 kb PstI fragment of the pac gene, which covers the middle region of PAC (Okahashi et al., 1989b), was radiolabelled as described above, and used as the probe.

RESULTS

Surface hydrophobicity and cell-surface PAC

Strains Xc and Yc freshly isolated from human dental plaque produced acid from sorbitol, mannitol, raffinose, inulin and melibiose, and did not produce ammonia from arginine, indicating that these strains belong to the species S. mutans (Hardie, 1986). Both strains possessed the serotype c antigen of Bratthall (1970). Strains Xc and Yc were repeatedly subcultured in BHI broth, and tested for their cell-surface hydrophobicity. The surface hydrophobicity of strain Xc markedly decreased after subculturing 60 times, whereas that of strain Yc remained unaltered after 100 subcultures (Fig. 1). The amount of cell-surface antigens with which anti-PAC serum reacted was determined by radioimmunoassay. The amount of cell-surface PAC of strain Xc decreased significantly after repeated subculturing, but that of strain Yc did not (Fig. 2). After subculturing 100 times in BHI broth, the culture of strain Xc was diluted and plated out on MS-SB agar. After 3 d of incubation, all colonies were isolated, and examined for cell-surface hydrophobicity and the amount of cell-surface PAC. Among 56 colonies tested, 51 exhibited lower cell-surface hydrophobicity than strain Xc (Fig. 3). The amount of cell-surface PAC of all colonies, except one, was less than that of the fresh isolate. There was a close correlation between the cell-surface hydrophobicity and the amount of cell-surface PAC (Fig. 3).

One relatively hydrophobic variant and one hydrophilic variant were isolated from the 100-fold subculture of strain Xc, and designated as Xc100H and Xc100L, respectively. Two hydrophobic variants were isolated from the 100-fold subculture of strain Yc, and designated as Yc100H1 and Yc100H2, respectively. No hydrophilic variant of strain Yc was found. Radioimmunoassay showed that production of cell-surface PAC by the hydrophilic variant Xc100L was lower than that of the antigen by strains Xc and Xc100H (Table 1). Strain Yc100H2 produced higher amounts of cell-surface PAC than strains Yc and Yc100H1. SDS-PAGE analysis of culture supernatants and cell extracts of fresh isolates and their subcultured variants are shown in Figs 4 and 5. Gels stained with Coomassie Brilliant Blue showed that culture supernatants and cell extracts of all the strains possessed a protein of M, 190 000 (PAC). Western blotting showed that anti-PAC serum reacted with the high-M, protein (data not shown). The amount of PAC in culture supernatant and cell extract of strain Xc100L was very small. On the other hand, culture supernatant and cell extract of strain Yc100H2 contained a large amount of PAC. Culture supernatant of strain Yc100H1 gave extra bands at the top of the gel after SDS-PAGE (Fig. 4, lane 5). These bands did not react with anti-PAC serum in Western blotting (data not shown).
Fig. 1. Effects of repeated subculturing of freshly isolated *S. mutans* strains on their cell-surface hydrophobicity. Two fresh isolates, strains Xc (○) and Yc (●), were subcultured in BHI broth. The relative surface hydrophobicities of every tenth subculture were determined by their adsorption to hexadecane. Values are the mean ± SD of triplicate assays.

Fig. 2. Effects of repeated subculturing of freshly isolated *S. mutans* strains Xc (○) and Yc (●) on their ability to produce cell-surface PAC. The amount of cell-surface PAC was determined by radioimmunoassay. Values are the mean ± SD of triplicate assays.

Fig. 3. Correlation between cell-surface hydrophobicity and cell-surface PAC of freshly isolated *S. mutans* strain Xc (○) and its 100-fold subcultured variants (●). The correlation coefficient of the data shown is 0.681.

**Adsorption to S-HA**

The ability of two fresh isolates and their subcultured variants to attach to S-HA was examined. The hydrophilic variant Xc100L attached in smaller numbers to S-HA than other hydrophobic strains (Table 1).
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Fig. 4. SDS-PAGE of culture supernatants of freshly isolated S. mutans strains and their subcultured variants. Culture supernatants were concentrated by ammonium sulphate precipitation, and analysed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The M, markers used were ovalbumin (M, 43000), catalase (60000), bovine serum albumin (67000), phosphorylase b (94000) and ferritin (220000). Lane 1, strain Xc; lane 2, strain Xc100H; lane 3, strain Xc100L; lane 4, strain Yc; lane 5, strain Yc100H1; lane 6, strain Yc100H2. The arrow indicates PAc of M, 190000.

Table 1. Hydrophobicity, cell-surface PAc production and adsorption to S-HA of fresh S. mutans isolates and their subcultured variants

The relative surface hydrophobicity of the cells was determined by their adsorption to hexadecane. The amount of cell-surface PAc was determined by radioimmunoassay. [3H]Thymidine-labelled bacteria (5 x 10⁷) were allowed to react with S-HA (20 mg) in 1 ml buffered KCl at 37 °C for 1 h. Values are the mean ± SD of triplicate assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hydrophobicity (%)</th>
<th>Cell-surface PAc (c.p.m.)</th>
<th>10⁻⁶ × No. of cells adsorbed per 20 mg S-HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xc</td>
<td>27.9 ± 5.4</td>
<td>6771 ± 835</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Xc100H</td>
<td>17.0 ± 5.0</td>
<td>4775 ± 540</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Xc100L</td>
<td>1.3 ± 1.6</td>
<td>2505 ± 376</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Yc</td>
<td>20.6 ± 4.0</td>
<td>8293 ± 922</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Yc100H1</td>
<td>19.7 ± 2.4</td>
<td>8273 ± 418</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Yc100H2</td>
<td>32.2 ± 4.5</td>
<td>9555 ± 635</td>
<td>7.3 ± 0.8</td>
</tr>
</tbody>
</table>

RNA dot-blot analysis and Southern hybridization

The expression of PAc-specific mRNA transcripts by strains Xc, Xc100H and Xc100L was examined by RNA dot-blotting. As shown in Fig. 6, the expression of PAc-specific mRNA by hydrophilic strain Xc100L was fourfold lower than that by its parent strain Xc and hydrophobic variant Xc100H. To examine whether a deletion or recombination of the pac gene occurred
Fig. 5. SDS-PAGE of cell extracts of freshly isolated *S. mutans* strains and their subcultured variants. Whole cells were suspended in 8 M-urea, 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, and heated at 100 °C for 3 min. The cell extracts were clarified by centrifugation, and analysed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. See Fig. 4 legend for contents of each lane.

Fig. 6. RNA dot-blot analysis of *pac* transcripts. Serial twofold dilutions of RNA (10 μg ml⁻¹) from strains Xc (lane 1), Xc100H (lane 2) and Xc100L (lane 3) were probed with the ³²P-labelled *pac* gene from plasmid pPC41.

during the repeated subculturing, the chromosomal DNAs from strains Xc, Xc100H and Xc100L were digested with *EcoR*I, *BamH*I, *PstI* or *HindIII*, and used for Southern blot analysis. No difference of size in the restriction fragments of chromosomal DNAs among these strains was detected (Fig. 7).
Fig. 7. Southern blot analysis of the pac gene. The $^{32}$P-labelled pac gene was used as the probe. Chromosomal DNAs from strains Xc (lane 1), Xc100H (lane 2) and Xc100L (lane 3) were digested with EcoRI (a), BamHI (b), PstI (c) or HindIII (d), electrophoresed on agarose gel, and transferred to nitrocellulose membranes. Hybridization was performed according to the procedure of Southern (1975).

DISCUSSION

Numerous bacterial pathogens exhibit both qualitative and quantitative variation of various cellular components after repeated subculturing. In this study we subcultured two fresh isolates, strains Xc and Yc, of S. mutans serotype c 100 times in BHI broth, and showed a marked decrease of cell-surface hydrophobicity in strain Xc after subculturing 60 times. Similar results were previously reported by Westergren & Olsson (1983). In contrast, the hydrophobicity of strain Yc remained almost unaltered after repeated subculturing. A variant Yc100H2 with increased hydrophobicity was isolated from the 100-fold subculture of strain Yc. These findings indicate that variants with decreased hydrophobicity and those with increased hydrophobicity emerge after repeated subculturing.

McBride et al. (1984) analysed, by SDS-PAGE, proteins in culture supernatants and cell extracts of fresh S. mutans isolates and the subcultured variants isolated by Westergren & Olsson (1983). They demonstrated that cell extracts of hydrophilic subcultured variants did not contain a number of high-$M_r$ (mainly $M_r$, 190000) proteins which are present on cells of hydrophobic fresh isolates. However, the high-$M_r$ proteins appeared in the culture supernatants of the variants, suggesting that the inability to find them in the cell wall was because they were not incorporated into the cell wall. On the other hand, our hydrophilic variant Xc100L had a reduced ability to produce both cell-free and cell-associated PAc. Recently, Okahashi et al. (1989a) reported that PAc-defective mutants constructed by inserting an erythromycin-resistance gene into the pac gene of S. mutans serotype c exhibit decreased cell-surface hydrophobicity compared with their parent strain. The present study shows a close correlation...
between cell-surface hydrophobicity and the amount of PAc on the cell surface, indicating that location of PAc on the streptococcal surface is important in the cell-surface hydrophobicity.

There is currently great interest in the role of cell-surface hydrophobicity in mediating bacterial attachment to salivary pellicles on teeth. Several investigators have shown a general correlation between the hydrophobicity of bacteria and their adherence to experimental pellicles (Gibbons & Etherden, 1983; Westergren & Olsson, 1983) or their colonization on human tooth surfaces in vivo (Svanberg et al., 1984). This was supported by our results that the hydrophilic variant attached in smaller numbers to S-HA than the hydrophobic strains.

Lee et al. (1988) and Okahashi et al. (1989a) have recently cloned the structural gene for a protein antigen of \( M \), 190000 (called P1 and PAc, respectively) of \( S. mutans \) serotype c. PAc is immunologically identical to antigens P1, B, I/II and IF (Forester et al., 1983; Ohta et al., 1989). Holt et al. (1982) and Holt & Ogundipe (1987), and Takahashi et al. (1989) have reported the cloning of a gene coding for a high-\( M \), protein antigen (called SpaA and PAg, respectively) of \( S. sobrinus \) serotype \( g \) that cross-reacts immunologically with PAc. The complete nucleotide sequence of the gene for PAc has been also determined (Okahashi et al., 1989b). The \( pac \) gene consists of 4695 bp and codes for a protein of \( M \), 170773. A potential promotor sequence and a putative Shine–Dalgarno sequence precede the open reading frame. Two internal repeating nucleotide sequences are present in the \( pac \) gene. From these findings the question has been raised as to whether or not a deletion or recombination of the \( pac \) gene, such as occurs with the genes for type 1 fimbriae of \( Escherichia coli \) (Abraham et al., 1985), pilin of \( Neisseria gonorrhoeae \) (Segal et al., 1985), protein II of \( N. gonorrhoeae \) (Stern et al., 1986) and group A streptococcal M protein (Hollingshead et al., 1987), will occur after repeated subculture. In this regard, Ueda & Kuramitsu (1988) have suggested that homologous recombination between the two homologous tandem genes coding for glucosyltransferases occurs in spontaneous mutants of \( S. mutans \) serotype c defective in sucrose-dependent colonization on smooth surfaces. However, no difference in the \( pac \) gene among strains Xc, Xc100H and Xc100L was observed by Southern blot analysis. On the other hand, RNA dot-blot analysis showed that the degree of expression of PAc-specific mRNA, as well as the amount of PAc produced, by strain Xc100L was lower than that by strains Xc and Xc100H. These results suggest that a mechanism which regulates the transcription of the \( pac \) gene may participate in quantitative variation of PAc after repeated subculture. The mechanism of transcription regulation for the \( pac \) gene is worthy of further study.

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REFERENCES


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