Molecular characterization of *Streptococcus mutans* strains isolated from the heart valve of an infective endocarditis patient

Hirotoshi Nemoto, Kazuhiko Nakano, Ryota Nomura and Takashi Ooshima

Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan

*Streptococcus mutans*, known to be an aetiological agent of dental caries, is occasionally isolated from patients with infective endocarditis (IE). *S. mutans* strains with a defect in all three types of glucosyltransferase (GTF) obtained from an infected heart valve extirpated from an IE patient have been reported previously. In this study, molecular analyses of strains detected in heart valve (strain V1) and dental plaque (strain P1) samples taken from the same patient were performed. Complete nucleotide alignments of the *gtfB*, *gtfC* and *gtfD* regions in strains V1 and P1, as well as in the reference strain MT8148, were determined, which revealed the existence of alignments with a high similarity to erythromycin- and spectinomycin-resistance genes in the middle of the *gtfB*, *gtfC* and *gtfD* genes, respectively, of V1. Strain V1 also showed a higher MIC for these two antibiotics compared with strain P1. Next, primers to detect the specific sequences of the antibiotic-resistance genes in strain V1 were constructed and PCR amplification was performed with template DNA from dental plaque and infected valve tissue samples taken from the patient. Attenuated expression of GTFs in V1 caused a significantly lower susceptibility to phagocytosis by human polymorphonuclear leukocytes compared with the reference strain. These results suggest that the blood isolate V1 found in the oral cavity invaded and survived in the bloodstream for a long duration and that this was related to its virulence in IE in our patient.

INTRODUCTION

*Streptococcus mutans* is known to be a major aetiological agent of dental caries, but is sometimes isolated from the blood of patients with bacteraemia and infective endocarditis (IE) (Moreillon & Que, 2004). The bacterium synthesizes adhesive glucan from sucrose by the action of three types of glucosyltransferase (GTF: GTFB, GTFC and GTFD), each of which, at an optimum ratio, has been shown to be important for sucrose-dependent adherence (Ooshima et al., 2001). GTFs are known to be important virulence factors of *S. mutans* for dental caries, as inactivation of the genes encoding each GTF results in a drastic reduction in caries-inducing activity in animal experiments (Yamashita et al., 1993).

We previously isolated *S. mutans* strains (including strain V1) that did not express GTFs from an IE patient (Nomura et al., 2006). The colony morphology of these strains on agar plates was apparently different from that of typical *S. mutans* strains, whereas it was similar to strains generated by inactivation of the genes encoding GTFs (Ooshima et al., 2001). As the occurrence of *S. mutans* without GTF expression is extremely rare in the oral cavity, we performed molecular characterization of the GTFs in the V1 strain isolated from the infected heart valve of this patient and speculate on its origin based on the results of strain-specific characterization.

METHODS

**Bacterial strains.** Table 1 summarizes the *S. mutans* strains used in this study. Strains V1–V7 and P1–P18 were isolated from heart valve and dental plaque specimens, respectively, obtained from an IE patient (Nomura et al., 2006). Strain MT8148 and its isogenic mutant strains defective in GTF expression (S2, R4, DE1, S5, B9, C13 and BC7s) were also used (Ooshima et al., 2001). All strains were grown in brain heart infusion broth (Difco Laboratories), as well as on Mitis Salivarius agar (Difco) with bacitracin (100 U ml⁻¹; Sigma) and 15 % sucrose. When culturing the isogenic mutant strains, appropriate antibiotics (10 μg erythromycin ml⁻¹ and/or 500 μg kanamycin ml⁻¹) were added for selection.

**Determination of the alignment of the genes encoding GTFs.** The nucleotide alignment of the genes encoding GTFs in strains V1 and P1 was determined in order to compare it with that in MT8148 (GenBank accession numbers for *gtfB*, *gtfC* and *gtfD* are D88651, D88652 and D88653, respectively; Fujisawa et al., 1998). Primers used to amplify the gene fragments were constructed as illustrated in Fig. 1. Table 2 lists all of the primers utilized to determine the complete sequences in this study. PCR was carried out in a 20 μl reaction...
mixture containing 2.5 U PrimeSTAR HS DNA polymerase (Takara Bio), 0.2 μM oligonucleotide primers, template DNA and 1.5 mM MgCl₂, according to the manufacturer’s protocol. The PCR products were separated by electrophoresis on a 0.7 % agarose gel and amplified DNA was extracted using a QIAEX II gel extraction kit (Qiagen). The extracted DNA was cloned directly into a pGEM-T Easy vector (Promega), and the sequence of the fragment was determined using a DNA sequencing system (ABI PRISM 310 Genetic Analyser). Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Features*</th>
<th>GTF expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1–V7</td>
<td>Clinical isolates from extirpated heart valve of an IE patient</td>
<td>– – –</td>
<td>Nomura et al. (2006)</td>
</tr>
<tr>
<td>P1–P18</td>
<td>Clinical isolates from dental plaque of an IE patient</td>
<td>+ + +</td>
<td>Nomura et al. (2006)</td>
</tr>
<tr>
<td>MT8148</td>
<td>Oral isolate from Japanese child patient</td>
<td>+ + +</td>
<td>Ooshima et al. (1983)</td>
</tr>
<tr>
<td>S2</td>
<td>EmR; strain MT8148 carrying erm inserted into gtfB</td>
<td>– + +</td>
<td>Ooshima et al. (2001)</td>
</tr>
<tr>
<td>R4</td>
<td>EmR; strain MT8148 carrying erm inserted into gtfC</td>
<td>+ – +</td>
<td>Ooshima et al. (2001)</td>
</tr>
<tr>
<td>DE1</td>
<td>EmR; strain MT8148 carrying erm inserted into gtfD</td>
<td>+ + –</td>
<td>Ooshima et al. (2001)</td>
</tr>
<tr>
<td>S5</td>
<td>EmR; strain MT8148 carrying erm inserted into both gtfB and gtfC</td>
<td>– – +</td>
<td>Ooshima et al. (2001)</td>
</tr>
<tr>
<td>B9</td>
<td>EmR KmR; strain DE1 carrying aphA inserted into gtfB</td>
<td>– + +</td>
<td>Ooshima et al. (2001)</td>
</tr>
<tr>
<td>C13</td>
<td>EmR KmR; strain DE1 carrying aphA inserted into gtfC</td>
<td>+ – –</td>
<td>Ooshima et al. (2001)</td>
</tr>
<tr>
<td>BC7s</td>
<td>EmR KmR; strain DE1 carrying aphA inserted into both gtfB and gtfC</td>
<td>– – –</td>
<td>Ooshima et al. (2001)</td>
</tr>
</tbody>
</table>

*erm, erythromycin-resistance gene; aphA, kanamycin-resistance gene; EmR, erythromycin resistant; KmR, kanamycin resistant.

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Fig. 1. Illustration of the nucleotide alignment of gtfB, gtfC and gtfD and their adjacent regions in strains MT8148, P1 and V1. Shown are the gtfB and gtfC regions (a) and the gtfD region (b). The same intensity of shading in the dark closed box corresponds to the region of high identity in each gene. Arrows in the rectangular boxes indicate the coding region of each gtf gene. Small arrows located below the rectangular boxes indicate primers used for determination of the sequence, whilst those inside the boxes indicate primers used for specification of an alignment similar to strain V1. Bold arrows indicate nucleotide alignments with a high identity to the genes encoding adenine methylase (erm) or spectinomycin adenyltransferase (add9).
V1 were constructed: BVF and BVR for the
nucleotide alignment of amplified fragments from each positive
specimen as described previously (Nakano et al., 2006a) and PCR analyses
constructed for this study were carried out. Bacterial DNA was
dextracted from each specimen as described previously (Nakano
et al., 2006), and 49 dental plaque specimens collected from patients referred to the
Department of Oral Surgery of Osaka Rosai Hospital prior to
cardiovascular surgery, were then examined. Bacterial DNA was
collected from patients who underwent operations at the
Department of Cardiovascular Surgery, Osaka Rosai Hospital, Sakai,
Aneurysms), collected from patients who underwent operations at the
protocol approved by the Ethics Committee of Osaka Rosai Hospital,
identification of specimens containing genetic features similar
to those of strains V1–V7. A total of 190
were carried out using the methods described above.

Identification of specimens containing genetic features similar
to those of strains V1–V7. Prior to our analysis, the sensitivity of the
PCR methods used to identify strains similar to V1–V7 was examined.
The detection limit was determined by simultaneous PCR assays using
known numbers of bacterial cells from strains V1–V7. A total of 190
cardiovascular specimens (112 from heart valves and 78 from aortic
aneurysms), collected from patients who underwent operations at the
Department of Cardiovascular Surgery, Osaka Rosai Hospital, Sakai,
Osaka, Japan, between December 2004 and July 2007, according to a
protocol approved by the Ethics Committee of Osaka Rosai Hospital,
and 49 dental plaque specimens collected from patients referred to the
Department of Oral Surgery of Osaka Rosai Hospital prior to
cardiovascular surgery, were then examined. Bacterial DNA was
dextracted from each specimen as described previously (Nakano et al.,
2006a) and PCR analyses constructed for this study were carried out.
The nucleotide alignment of amplified fragments from each positive
specimen was confirmed by sequencing analysis.

Table 2. PCR primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1F</td>
<td>GTGGTGGTACTCCCTGTAATAA</td>
</tr>
<tr>
<td>B1R</td>
<td>AGAAACCTTGAAGGTTCCGATAGG</td>
</tr>
<tr>
<td>B2F</td>
<td>CGGTTGCCATGACAGTGAATG</td>
</tr>
<tr>
<td>B2R</td>
<td>ACAAGGCAACATGTATCGTAT</td>
</tr>
<tr>
<td>C1F</td>
<td>GGGTTTCTGAGTTAGGAGTCTT</td>
</tr>
<tr>
<td>C1R</td>
<td>AAGGTTATTGCTCTTCAATC</td>
</tr>
<tr>
<td>C2F</td>
<td>GGGTTCAACGATGGCAATCAC</td>
</tr>
<tr>
<td>C2R</td>
<td>GCTTACAATTTGTAAGCATTG</td>
</tr>
<tr>
<td>BC1F</td>
<td>CTGTGACTTGGTACGCCCAAC</td>
</tr>
<tr>
<td>BC2F</td>
<td>TTAGCGTCTGCCCTATAGTTA</td>
</tr>
<tr>
<td>BC2R</td>
<td>CATAGGCGTTTTGGATACAGAAT</td>
</tr>
<tr>
<td>BC3F</td>
<td>AGTTACAAAGCTCGGCCCAT</td>
</tr>
<tr>
<td>BC3R</td>
<td>TCTTTTGAAGACGGCTAG</td>
</tr>
<tr>
<td>D1F</td>
<td>GGTGCAATTCAAGCAGAAAGAA</td>
</tr>
<tr>
<td>D1R</td>
<td>CAAGAAGGCCTGCAAGACCT</td>
</tr>
<tr>
<td>D2F</td>
<td>TCCGACTATGGAAAAAGCC</td>
</tr>
<tr>
<td>D2R</td>
<td>AAGGAAGTACCCCAAGATTG</td>
</tr>
<tr>
<td>D3F</td>
<td>AGCAAGAATAAGGGCAAAATGTC</td>
</tr>
<tr>
<td>D3R</td>
<td>TGGCAATAGCTTCTCATGCTGGA</td>
</tr>
<tr>
<td>BVF</td>
<td>GGGGACGCCCATAAAGACC</td>
</tr>
<tr>
<td>BVR</td>
<td>GACAGCTCIAAGGAGCTAA</td>
</tr>
<tr>
<td>DVF</td>
<td>GCGCTTACTGGTCTTATATG</td>
</tr>
<tr>
<td>DVR</td>
<td>GATTAGTACCATATTTATCTCAG</td>
</tr>
</tbody>
</table>

Phagocytosis assay. Phagocytosis susceptibility was evaluated using a
method described previously (Nakano et al., 2004). Strain MT8148
and its GTF-deficient isogenic mutants (strains S2, R4, DE1, S5, B9,
C13 and BC7s; Table 1), as well as strains P1 and V1, were analysed.

RESULTS AND DISCUSSION

Examination of blood isolates of S. mutans from patients
with bacteremia or IE has been reported in several studies
in which alterations of cell-surface components such as
serotype-specific polysaccharides or protein antigens were
described (Munro & Macrina, 1993; Fujiwara et al., 2001;
Chia et al., 2004; Nomura et al., 2006, 2007; Nakano et al.,
2007). For GTFs, analysis of an S. mutans isogenic mutant
strain with defects in the three types of GTF generated from
the blood isolate strain V403 showed that sucrose-derived
expolysaccharides contributed to infectivity in endocarditis
caused by S. mutans (Munro & Macrina, 1993). However, S. mutans
strains lacking expression of all three types of GTF have also been isolated from infected heart
vascular tissues (Nomura et al., 2006), indicating that GTFs do
not contribute directly to the virulence of S. mutans in IE.
In contrast, Shun et al. (2005) demonstrated that the survival rate of mice infected with an isogenic mutant
strain lacking all three types of GTF was significantly lower
than that of those infected with the parental strain as well as
the uninfected control group.

The homologous regions of gtfB, gtfC and gtfD in MT8148,
P1 and V1 are illustrated in Fig. 1. The alignment of gtfB–
gtfC in strain P1 was almost identical to that in MT8148.
However, regions corresponding to nt 3150–5400 from the
start of MT8148 and P1 were not identified in strain V1.
Furthermore, a BLAST search revealed that an approximately
670 nt region in this 2250 nt area of V1 was highly
homologous (99 % identity) to the nucleotide sequence of
the adenine N6 methyltransferase (ermB) gene of Streptococcus pneumoniae (GenBank accession no.
DQ855644). For gtfD, nucleotide identity between
MT8148 and P1 was found throughout the entire length.
In contrast, an approximately 1200 bp insertion
was identified in gtfD of strain V1 at a location corresponding
to nt 2100 of MT8148 and P1. A BLAST search revealed that
the inserted fragment was highly homologous (97 % identity)
to spectinomycin adenyltransferase (add9) of Enterococcus faecalis (GenBank accession no.
M69221). These findings explain why no expression was detected for
any of the three types of GTF in strain V1.

RT-PCR analysis revealed mRNA expression of the two
antibiotic-resistance genes in strains V1–V7 (data not shown).
We previously found that the MIC for erythromycin of strains V1–V7 was greater than 24 µg ml⁻¹,
which was significantly higher than that for strains P1–P18 (0.96 µg ml⁻¹) and MT8148 (0.06 µg ml⁻¹), as determined
by a macrodilution broth method (Nomura et al., 2006).
For spectinomycin, the MIC for strains V1–V7 was greater than
1600 µg ml⁻¹, which was significantly higher than
that for strains P1–P18 (6.4 µg ml⁻¹) and MT8148 (6.4 µg
erythromycin was greater than 24 μg ml⁻¹ due to the existence of an erythromycin-resistance gene cassette. In contrast, the MICs of spectinomycin for the GTF-defective strains were similar to that for MT8148.

We previously analysed the association of phagocytosis susceptibility by human polymorphonuclear leukocytes and surface protein antigens of S. mutans, which showed that a defect in GTFD led to a significantly lower phagocytosis rate (Nakano et al., 2006b). In the present study, strains defective in at least two types of GTF were analysed, each of which showed a significantly lower phagocytosis rate than the parental strain (P < 0.001), with the lowest rate shown by the strain that lacked all three types (Fig. 2). The mean phagocytosis rates of strains P1 and V1 were 74.0 ± 2.4 % and 68.4 ± 2.2 %, respectively. In another previous study, we analysed the phagocytosis susceptibility of 20 clinical strains using the same method, which revealed a mean rate of 82.6 ± 6.1 % (Nomura et al., 2004). The phagocytosis rate for V1–V7 (73.3 ± 4.0 %) in the present study was significantly lower than the mean for these 20 clinical strains (P < 0.01; Mann–Whitney U-test), indicating that a lack of all three types of GTF leads to low antigenicity, enabling the strains to survive in the blood for a longer duration.

Next, we attempted to determine the origin of strain V1 isolated from the infected heart valve. One possibility is that the strain was present in the oral cavity and then invaded the bloodstream, whilst another is that the surface antigen was altered after invasion into the bloodstream. In order to analyse these two possibilities, we constructed a PCR method for identification of strains V1–V7. Two sets of primers were designed based on the specific nucleotide alignment of the gtfB and gtfD regions of strain V1 (Fig. 1).

Amplified fragments obtained by this method were a 355 bp region for gtfB and a 407 bp region for gtfD. PCR analysis showed that positive bands were identified not only in the heart valve specimen, but also in the dental plaque sample (Fig. 3). As the detection limit of this system was calculated to be 10–100 c.f.u. per specimen, the dental plaque sample from our IE patient contained at least that number of strains similar to V1. Based on these results, we consider it highly possible that the V1 strain was first present in the oral cavity and then invaded the bloodstream, which led to the development of IE.

We have been conducting studies to determine the frequency of detection of oral bacteria in cardiovascular specimens (Nakano et al., 2006a, 2007) and were interested in the clinical distribution frequency of strains similar to V1–V7. Thus the presence of these strains in cardiovascular specimens, including heart valve and atheromatous plaque samples extirpated during surgery, and dental plaque samples obtained during oral examination were analysed using the PCR method constructed for this study. There were no positive bands produced by the template DNA extracted from the heart valve specimens and dental plaque samples. However, two out of 78 aneurysmal specimens (from an 80-year-old male and an 84-year-old female) showed a positive reaction using the DVF/DVR primer set, although they were negative for the primers BVF/BVR. These results indicate that the distribution rate of strains similar to V1–V7 is extremely low among oral strains as well as in cardiovascular specimens.

In summary, the absence of GTF expression due to the insertion of antibiotic-resistance genes into the gtf genes of strains V1–V7 isolated from an IE patient resulted in their lower susceptibility to phagocytosis and the acquisition of antibiotic resistance, which may contribute to the virulence of S. mutans in IE. The distribution rate of these strains is considered to be extremely low; however, their existence should be kept in mind when investigating clinical strains.

Fig. 2. Phagocytosis rates of MT8148 and its isogenic mutant strains defective in GTFs. Letters in parentheses and shading indicate the number of GTFs expressed in each strain. There were significant differences between MT8148 and the other strains revealed by Fisher’s protected least significant difference analysis. ** P < 0.01; *** P < 0.001.

Fig. 3. Results of a PCR assay to detect strains with a nucleotide alignment similar to that of strain V1. Bacterial DNA was extracted from strains V1 (lane 1) and P1 (lane 2), as well as from heart valve (lane 3) and dental plaque (lane 4) samples obtained from an IE patient from whom V1 was isolated. M, Molecular size marker (100 bp DNA ladder).
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REFERENCES


