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Development of a PCR Method for Rapid Identification of New *Streptococcus mutans* Serotype *k* Strains

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In a previous study, we isolated and characterized a new serotype *k* of *Streptococcus mutans* from human blood and oral cavities. Analysis of the genes involved in biosynthesis of the serotype-specific polysaccharide of serotype *k* strains revealed that the serotype *k*-specific nucleotide alignment was commonly present in the 5′ region of the *rgpF* gene (350 bp from the initial sequence) compared to the reference strains, and then a method for rapid identification of serotype *k* strains was developed by use of PCR with primers designed on the basis of the sequence of the variable region. PCR assays with primers specific for amplification of serotype *k* strains showed a negative reaction with serotype *c*, *e*, and *f* strains and a positive reaction with serotype *k* strains, with the sensitivity for identification of the serotype *k* strains shown to range from 5 to 50 cells. Next, the frequency of positive reactions for serotype *k*-specific primers was surveyed with DNA taken from saliva samples from 200 subjects (2 to 18 years of age), and 10 of those showed a positive reaction, which was higher than the frequency in our previous survey with a serological method. In addition, all saliva samples from subjects with serotype *k* strains in our previous study were shown to be positive with the serotype *k*-specific primers. These results indicate that this new PCR method is effective for identification of subjects with *S. mutans* serotype *k*.

Streptococcus mutans, which is known to be a major cariogenic bacterium as well as one of the pathogens involved with bacteremia and infective endocarditis (IE), was previously classified into three serotypes, *c*, *e*, and *f*, based on the chemical composition of serotype-specific polysaccharides (5). The serotype-specific polysaccharide was shown to be composed of rhamnose-glucose polymers, with a backbone of rhamnose and side chains of α- or β-linked glucosidic residues (7). The genes involved in the synthesis of serotype-specific rhamnose-glucose polymers have also been cloned and sequenced. Four *rml* genes (*rmlA* through *rmlD*) are related to the synthesis of dTDP-L-rhamnose (18, 19), and the *gluA* gene encodes the enzyme that catalyzes the production of the immediate precursor of the glucose side chain donor (23). In addition, the six genes (*rgpA* through *rgpF*) required for synthesis of rhamnose-glucose polymers have been cloned and sequenced (15, 24). Further, the *rgpG* gene has been implicated in the initiation of synthesis of rhamnose-glucose polymers (25).

In our previous study, *S. mutans* strains with a low amount of the glucose side chain in serotype-specific polysaccharide were defined as a new serotype, serotype *k*, which was estimated to have a distribution in the oral cavity of approximately 2% (10). Recently, PCR methods were developed to identify serotypes *c*, *e*, and *f* by using DNA extracted from saliva samples with primers constructed on the basis of the differences in the sequences of the region downstream of *rgpF* among each of the serotypes (16). However, there is no information for serotype *k* strains regarding the genes involved in formation of the side chain of the rhamnose backbone. In the present study, we analyzed those genes from serotype *k* strains and developed a PCR method to identify subjects with *S. mutans* serotype *k*.

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MATERIALS AND METHODS

***S. mutans* strains.** Table 1 lists the *S. mutans* strains used in this study. Blood isolates TW295 (*k*) and TW871 (*k*) (3) and orally isolated strain MT8148 (*c*) were selected from the stock culture collection in our laboratory (8). One hundred strains of *S. mutans* isolated from 100 children, which included 78 serotype *c*, 17 serotype *e*, 3 serotype *f*, and 2 serotype *k* (strains FT1 and SU1) strains (NN2000 series of isolates) (10), were also analyzed, as was another serotype *k* strain, YK1 (10). Strains AT1 and YT1, isolated in the present study, were confirmed as *S. mutans* according to the results of a double immunodiffusion method that utilized autoclaved extracts and serotype *k*-specific antiserum, which has been described previously (10). The specificity of the primers used was tested against the following organisms: *Streptococcus sanguinis* ATCC 10556, *Streptococcus oralis* ATCC 10557, *Streptococcus gordonii* ATCC 10558, *Streptococcus mitis* ATCC 903, *Streptococcus milleri* NCTC10703, and *Streptococcus salivarius* HHT.

Sequence analysis of genes involved in biosynthesis of serotype-specific polysaccharide. The *rgp* genes (*rgpA* through open reading frame 11 [ORF11]; total of 15,890 bp) were divided into six fragments. Each gene fragment was amplified by PCR with primers constructed on the basis of the nucleotide alignment of strain Xc (GenBank accession no. AB010970) or strain UA159 (GenBank accession no. AE014133) with LA*Taq* Polymerase (Takara Shuzo, Otsu, Japan). The PCR products were separated by electrophoresis on a 0.7% agar gel, and the amplified DNA was extracted with QIAEX (Qiagen, Düsseldorf, Germany). The DNA was directly cloned into a pGEM-T Easy vector (Promega, Madison, Wis.), for which the nucleotide sequence was determined by a dye-terminator reaction with a DNA sequencing system (373-18 DNA sequencer; Applied Biosystems, Foster City, Calif.) and an ABI PRISM cycle sequencing kit. Data analysis was performed with Gene Works software (IntelliGenetics, Mountain View, Calif.), and a multiple-alignment analysis was carried out with CLUSTAL W from the DDBJ (Mishima, Japan) (17). The nucleotide alignments of the *rgp* genes in TW295 (serotype *k*), TW871 (serotype *k*), FT1 (serotype *k*), and YT1 (serotype *k*) were compared with that of MT8148 (serotype *c*). The *rgp* genes of strain Xc (serotype *c*), for which the *rgp* genes were originally sequenced, and those of strain UA159 (serotype *c*) (1), of which the complete genome has been sequenced, were also compared with those of the serotype *k* strains.

Clinical specimens. Expectorated whole saliva (approximately 1 ml in each sample) was collected from 200 children and adolescents (2 to 18 years of age;

TABLE 1. *S. mutans* strains used in the present study

Strain	Serotype	Description	Reference
MT8148	*c*	Oral isolate from a healthy child	8
NN2001	*c*	Oral isolate from a 6-yr-old boy	10
NN2002	*e*	Oral isolate from a 9-yr-old boy	10
NN2003	*f*	Oral isolate from a 7-yr-old girl	10
TW295	*k*	Blood isolate from a 59-yr-old male with bacteremia following a tooth extraction	3
TW871	*k*	Blood isolate from a 45-yr-old female with infective endocarditis complicated by subarachnoid hemorrhage	3
FT1 (NN2011)	*k*	Oral isolate from a healthy 3-yr-old girl	10
SU1 (NN2029)	*k*	Oral isolate from a healthy 10-yr-old girl	10
YK1	*k*	Oral isolate from a healthy 7-yr-old girl with Down's syndrome	10
YT1	*k*	Oral isolate from a healthy 6-yr-old boy	This study
AT1	*k*	Oral isolate from a healthy 7-yr-old girl	This study

mean age, 7.9 ± 3.6 years) who visited the Pedodontics Clinic of Osaka University Dental Hospital in January and February 2004. Collection of the clinical specimens was carried out in accordance with the Osaka University Health Guideline for Studies Involving Human Subjects. The saliva samples were processed for the PCR assay by the method reported by Hoshino et al. (6) with some modifications. Briefly, nonstimulated whole saliva was collected in a sterile tube and kept on ice. Bacterial cells were collected in a microcentrifuge tube from 500 μl of saliva at 16,000 × *g* for 5 min, then treated in a microwave oven at 500 W for 5 min, and digested in *N*-acetylmuramidase SG (Seikagaku Corp., Tokyo, Japan) at 50°C for 1 h. Then, 80 μl of nuclei lysis solution (Promega) was added and incubated at 80°C for 5 min, followed by the addition of 60 μl of protein precipitation solution (Promega). The proteins were removed by centrifugation at 16,000 × *g* for 3 min, and the DNA was purified by phenol-chloroform extraction and ethanol precipitation. The extracted DNA was then dissolved in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

Development of PCR method for identification of serotype *k* strains. Table 2 lists the PCR primers used in this study. The forward primer (CEFK-F) was common for all of the serotypes and designed within the 3′ end region of *rgpE*, whereas the serotype *c*-, *e*-, and *f*-specific (non-serotype *k*) and serotype *k*-specific reverse primers (CEF-R and K-R, respectively) were designed within the serotype *k*-specific 5′ region of the *rgpF* gene (350 bp from the initial sequence) (Fig. 1). The specificities of the prospective primers were tested by the program Amplify (2), based on the DNA sequence information stored in GenBank EMBL. All of the primers were commercially synthesized (Proligo Japan, Kyoto, Japan).

PCR amplification was performed in a total volume of 20 μl with 1 μl of template solution and Ampli*Taq* Gold polymerase (Applied Biosystems) according to the manufacturer's instructions. The PCR amplification reaction was performed in a thermal cycler (iCycler; Bio-Rad, Hercules, Calif.) with the following cycling parameters: an initial denaturation at 95°C for 4 min and then 30 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR products were subjected to electrophoresis

TABLE 2. PCR primers used in the present study

Primer	Sequence (5′–3′)	Location[a]
CEFK-F[b]	ATT CCC GCC GTT GGA CCA TTC C	6236–6257
CEF-R[c]	CCG ACA AAG ACC ATT CCA TCT C	6508–6529
K-R[d]	CCA ATG TGA TTC ATC CCA TCA C	6508–6529

[a] The numbers correspond to the locations of the nucleotide sequences obtained from GenBank accession number AB010970 (strain Xc).
[b] Forward primer for serotype *c*, *e*, *f*, and *k S. mutans*.
[c] Reverse primer for serotype *c*, *e*, and *f S. mutans*.
[d] Reverse primer for serotype *k S. mutans*.

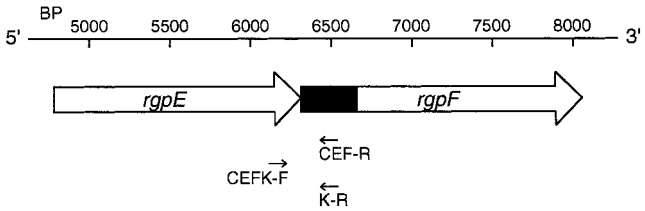

FIG. 1. Illustration of the serotype *k*-specific nucleotide region and location of the primers constructed for detection of serotype *k* strains by PCR. A base pair (BP) scale is shown above the map. The black box indicates the serotype *k*-specific nucleotide alignment region in the *rgpF* gene. The nucleotide number corresponds to the *rgpF* sequence in strain Xc obtained from GenBank (accession number AB010970).

in a 1.5% agarose gel-Tris-acetate-EDTA buffer. The gel was stained with 0.5 μg of ethidium bromide per ml and photographed under UV illumination. A 100-bp DNA ladder (New England BioLabs, Beverly, Mass.) was used as the molecular size standard.

First, the sensitivity of the PCR assay was determined by using titrated cultures of *S. mutans* MT8148 (serotype *c*), TW295 (serotype *k*), and FT1 (serotype *k*). The CEFK-F and CEF-R sets of primers were used for amplification of the genomic DNA of MT8148, and the CEFK-F and K-R sets were used for amplification of that of TW295 and FT1, respectively. The detection limit for simultaneous PCR was determined by using the known numbers of bacterial cells diluted in sterile distilled water. Genomic DNA extracted from strains MT8148 (serotype *c*), NN2001 (serotype *c*), NN2002 (serotype *e*), NN2003 (serotype *f*), TW295 (serotype *k*), TW871(serotype *k*), FT1 (serotype *k*), and YT1 (serotype *k*) was analyzed to confirm the specificity of the primers designed to classify the non-serotype *k* or serotype *k* type strains. In addition, the specificity of the primers was confirmed with genomic DNA from 100 *S. mutans* strains (78 serotype *c*, 17 serotype *e*, 3 serotype *f*, and 2 serotype *k* strains) previously isolated (NN2000 series of isolates) (10). After analyses of the sensitivity and specificity of the present method, 200 DNA samples extracted from the saliva of the 200 subjects were analyzed.

Analysis of subjects with serotype *k* strains identified previously. Saliva samples were collected from two subjects from whom FT1 (serotype *k*) or YK1 (serotype *k*) had been isolated from plaque samples in our previous study. DNA from these samples was collected by the method described above and subjected to PCR with the serotype *k*-specific primers.

RESULTS

Nucleotide alignment of *rgp* genes from *S. mutans* serotype *k* strains. The nucleotide alignments of *rgpA*, *rgpB*, *rgpC*, *rgpD*, *rgpE*, *rgpF*, ORF7, *rgpH*, *rgpI*, ORF10, and ORF11 of TW295 (serotype *k*), TW871 (serotype *k*), FT1 (serotype *k*), and YT1 (serotype *k*) were compared with those of MT8148 (serotype *c*), Xc (serotype *c*), and UA159 (serotype *c*). There were no significant differences in the putative amino acid sequences of RgpA, RgpC, RgpE, RgpH, ORF10, and ORF11 among all of the test and reference strains. On the other hand, 59 amino acids in the C-terminal region were estimated to be deleted in RgpB of strains FT1 (serotype *k*), YT1 (serotype *k*), and MT8148 (serotype *c*). In addition, 23 amino acids were estimated to be deleted in RgpD of TW871 (serotype *k*). As for ORF7, strains FT1 (serotype *k*) and YT1 (serotype *k*) were estimated to be composed of 603 and 675 amino acids, respectively, which were fewer than any of the other tested strains (803 amino acids). Further, 26 amino acids were estimated to be deleted in one-third of the N-terminal region of RgpI in strain FT1 (serotype *k*). The most prominent difference among the genes in each of the tested serotype *k* strains commonly compared to strains MT8148 (serotype *c*), Xc (serotype *c*), and UA159 (serotype *c*) was identified in the 5′ region of the *rgpF* gene (350 bp from the initial sequence) (Fig. 2). Thus, primers

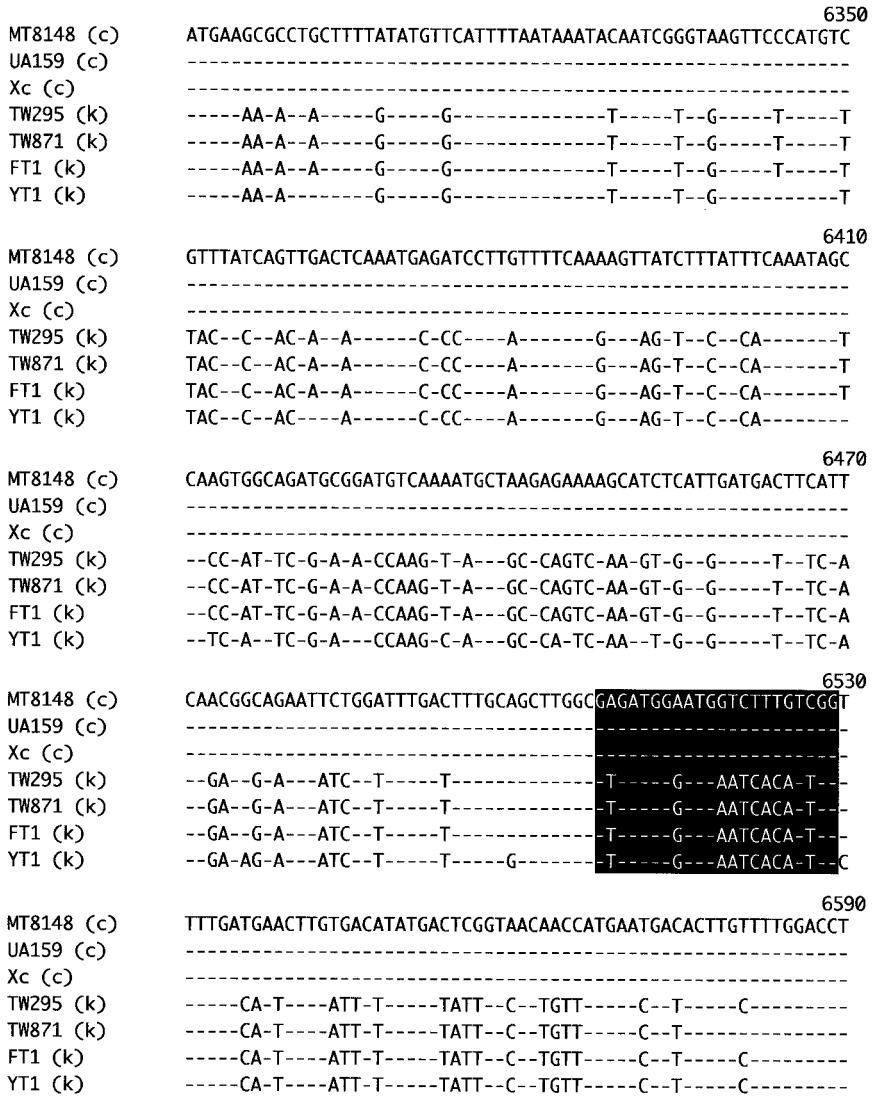

FIG. 2. Multiple-sequence alignment of the serotype *k*-specific 5′ region in the *rgpF* gene compared to that of the reference strains. Only nucleotides different from MT8148 are shown. Primers used for detection of serotype *k* and non-serotype *k* strains were constructed on the basis of the differences in nucleotide alignment (highlighted by the black box). Numbers above the nucleotides are those that appear in GenBank under accession number AB010970 (strain Xc). Serotypes are indicated in parentheses.

specific for the detection of serotype *k* strains were constructed on the basis of those sequences.

Specificity and sensitivity of the PCR assay. The PCR assay used in the present study showed positive bands with a non-serotype *k*-specific set of primers with MT8148 (serotype *c*) and with a serotype *k*-specific set of primers with TW295 (serotype *k*) and FT1 (serotype *k*), each of which produced single bands with the expected size of 294 bp, as assessed by electrophoresis (Fig. 3). The detection limit was determined in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was found to be between 5 and 50 cells for the serotype *k*-specific set of primers with strains TW295 (serotype *k*) and TW871 (serotype *k*) and between 50 and 500 cells for the non-serotype *k*-specific set of primers with strain MT8148 (serotype *c*). Non-serotype *k*-specific PCR products were selectively identified from among the serotype *c*, *e*, and *f* strains (listed in Table 1), and serotype *k*-specific PCR products were selectively detected from among the serotype *k* strains (Fig. 4). Positive bands were identified among the *S. mutans* strains while the other species showed negative reactions. The specificity of the primers was also confirmed with 100 strains (NN2000 series of isolates), which indicated that the PCR method utilized was valid.

Samples from subjects with a positive reaction to serotype *k*-specific primers. Figure 5 shows the results from saliva samples of representative subjects. Of the 200 samples, 190 showed a positive reaction to the non-serotype *k*-specific set of primers (specific for serotypes *c*, *e*, and *f*) but not to the serotype *k*-specific set, whereas 10 samples showed positive reactions to both primer sets. These 10 subjects included 3 males and 7 females, none of whom had heart disorders. In addition, saliva samples previously taken from two subjects from whom the serotype *k* strains YK1 and FT1 were isolated also showed a positive reaction to the serotype *k*-specific primers. In addition,

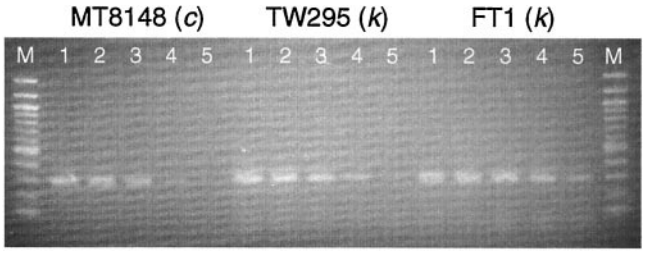

FIG. 3. Sensitivity of PCR assays for detection of non-serotype *k* and serotype *k* strains of *S. mutans*. The sensitivity of our PCR method was examined by using titrated cultures with 10^8 cells per ml from strains MT8148 (serotype *c*), TW295 (serotype *k*), and FT1 (serotype *k*). PCR was performed with the CEFK-F and CEF-R primer sets for MT8148 while the CEFK-F and K-R primer sets were used for TW295 and FT1. The detection limit for simultaneous PCR was determined by using the known numbers of bacterial cells diluted in sterile distilled water. The following numbers of cells were added: 5×10^4 (lanes 1), 5×10^3 (lanes 2), 5×10^2 (lanes 3), 5×10 (lanes 4), and 5 (lanes 5). M, molecular size marker (100-bp DNA ladder).

the use of a double immunodiffusion method for confirmation of serotype *k* strains AT1 and YT1, isolated from a subject whose saliva showed a positive reaction to serotype *k*-specific primers, also positively reacted to the serotype *k*-specific primers.

DISCUSSION

S. mutans is known to be a major causative bacterium of dental caries in humans and is occasionally isolated from the blood of the patients with IE (20, 21); however, its mechanism of invasion and survival in blood remains to be clarified. In a previous study (10), *S. mutans* serotype *k* strains were shown to

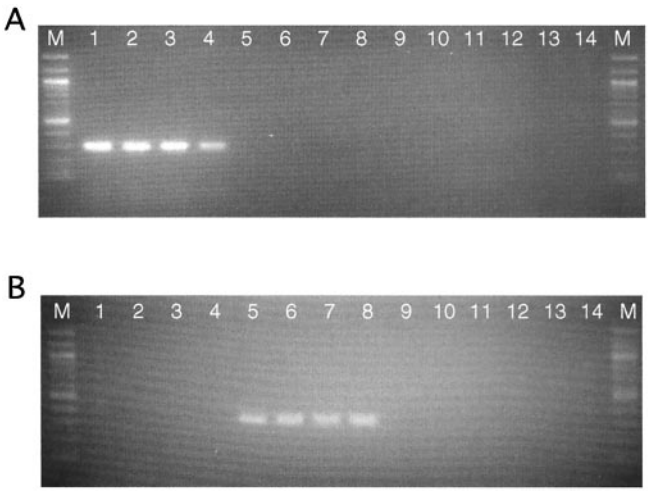

FIG. 4. PCR assay for the detection of *S. mutans* serotype *k* strains. (A) The primer sets CEFK-F and CEF-R were used for the detection of serotype *c*, *e*, and *f* strains. (B) The primer sets CEFK-F and K-R were used for the detection of serotype *k* strains. Lane 1, MT8148 (serotype *c*); lane 2, NN2001 (serotype *c*); lane 3, NN2002 (serotype *e*); lane 4, NN2003 (serotype *f*); lane 5, TW295 (serotype *k*); lane 6, TW871 (serotype *k*); lane 7, FT1 (serotype *k*); lane 8, YT1 (serotype *k*); lane 9, *S. sanguinis* ATCC 10556; lane 10, *S. oralis* ATCC 10557; lane 11, *S. gordonii* ATCC 10558; lane 12, *S. mitis* ATCC 903; lane 13, *S. milleri* NCTC10703; lane 14, *S. salivarius* HHT.

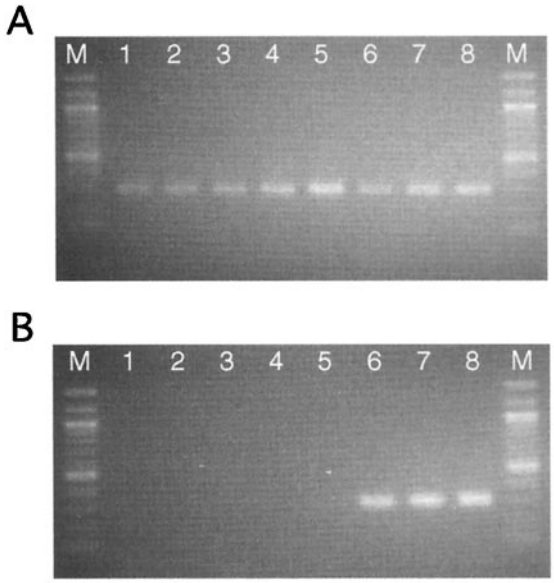

FIG. 5. Identification of subjects with *S. mutans* serotype *k* in saliva samples by PCR. (A) The primer sets CEFK-F and CEF-R were used for the detection of serotype *c*, *e*, and *f* strains. (B) The primer sets CEFK-F and K-R were used for the detection of serotype *k* strains. Lanes 1 through 5, samples from five representative subjects with a negative reaction to the serotype *k*-specific primers. Lanes 6 through 8, samples from three representative subjects with a positive reaction to the serotype *k*-specific primers. Samples in lanes 6 through 8 also reacted positively to the serotype *c*-, *e*-, and *f*-specific primers.

be less susceptible to phagocytosis, which indicated that this newly elucidated serotype may be one of the risk factors of IE caused by the bacterium. To prevent the occurrence of IE, host risk factors, such as congenital heart failure and acquired valve replacement, have been the focus of clinical practitioners. However, it is also important to elucidate which pathogenic bacteria have a potential to cause bacteremia and the ensuing IE. Microbiological diagnoses of bacteria in blood are generally based on conventional blood culturing, although molecular diagnoses have also been performed recently (4). PCR methods with primers constructed based on the 16S rRNA alignment are widely utilized for their rapid and sensitive detection of bacteremia (13). With a PCR assay, not only can the presence of bacteria in blood be identified but also the bacterial species itself can be specified, even in cases of culture-negative IE (22). To identify *S. mutans* by PCR, several methods have been proposed (6, 11, 14) and a PCR technique for differentiating between serotypes *c*, *e*, and *f* has been developed (16). In the present study, we utilized a PCR method to identify *S. mutans* serotype *k* strains.

The frequency of subjects with serotype *k* in the present study was 5%, which was higher than that (2%) found when analyzing a single representative strain from a single subject by an immunodiffusion method in a previous study (10). The detection limit of the present PCR method was shown to be from 5 to 50 cells (Fig. 3), indicating that subjects with a small number of the serotype *k* strain bacteria could be identified as positive. We observed positive reactions by the serotype *k* blood and oral isolates, as well as negative reactions by the 98 *c*, *e*, and *f* serotype strains by using the present serotype *k*-

specific set of primers. Further, analyses of saliva samples taken from two subjects with previously isolated serotype *k* strains (FT1 and YK1) (10) revealed positive reactions to the primers, as did two other serotype *k* strains (AT1 and YT1). As noted above, since serotype *k* strains are known to be less susceptible to phagocytosis by polymorphonuclear leukocytes (10), it is important to identify patients with serotype *k* strains, especially those regarded as having a risk for IE and who are receiving medical or dental treatment. We propose that the PCR method elucidated here will be beneficial for screening subjects with *S. mutans* serotype *k* strains.

The *rgpE*, *rgpH*, and *rgpI* genes have been shown to be correlated to glucose side chain formation in the serotype-specific polysaccharide of *S. mutans* (12, 24), however, there were no significant differences in the nucleotide alignment of these genes found in the present serotype *k* strains. On the other hand, the *rgpB*, *rgpD*, ORF7, and *rgpI* genes were shown to be altered in one or two serotype *k* strains, though such alterations were not commonly detected in all of the serotype *k* strains tested. In contrast, the 5′ region of the *rgpF* gene (350 bp from the initial sequence) was shown to be specific for the serotype *k* strains compared to the reference strains MT8148, Xc, and UA159, and we used it to develop the present PCR method for identification of serotype *k* strains. It is reasonable to speculate with high probability that there is a high possibility that *rgpF* itself is associated with glucose side chain formation in the serotype-specific polysaccharide of *S. mutans*. In order of level of involvement, RgpA, RgpB, and RgpF, encoded by the *rgpA*, *rgpB*, and *rgpF* genes, respectively, were each shown to be involved in the biosynthesis of the rhamnose backbone (16, 24). Therefore, we also considered that a variation of the *rgpF* gene may result in a short rhamnose backbone, causing a shortened attachment location of the glucose side chain. On the other hand, expression of the *rgpF* gene of MT8148 in the serotype *k* strains did not produce the properties of serotypes *c*, *e*, and *f* (data not shown), implicating the presence of other genes involved in biosynthesis of the glucose side chain. The functions of those genes in the rhamnose backbone of the O polysaccharide of *Escherichia coli* have been studied; however, the mechanisms in *S. mutans* remain to be elucidated in further molecular biological studies.

Our laboratory previously demonstrated that a serotype *k* blood isolate of TW871 showed a lower level of sucrose-independent adherence to saliva-coated hydroxyapatite than MT8148, as well as less cariogenicity in experiments with the caries-inducing rat model (9). We hypothesized that an alteration of glucan-binding protein C in TW871 caused the reduction of in vitro adhesion and the caries scores in vivo. However, the caries-inducing activities of TW871 have not been discussed in terms of alteration of the serotype-specific polysaccharide compared with that in MT8148. By utilizing a mutant strain without a glucose side chain in the serotype-specific polysaccharide as well as wild-type serotype *k* strains, the unknown function related to dental caries may be elucidated in further studies.

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