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Phylogenetic analyses and detection of viridans streptococci based on sequences and denaturing gradient gel electrophoresis of the rod shape-determining protein gene

Ikuri Konishi[1], Tomonori Hoshino[1]*, Yoshio Kondo[1,2], Kan Saito[1], Miyuki Nishiguchi[1], Kyoko Sato[1] and Taku Fujiwara[1]

[1]Department of Pediatric Dentistry, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [2]Division of Microbiology and Oral Infection, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Background: Population analysis of viridans streptococci is important because these species are associated with dental caries, bacteremia, and subacute endocarditis, in addition to being important members of the human oral commensal microbiota.

Design: In this study, we phylogenetically analyzed the rod shape-determining protein gene (*rodA*), which is associated with cellular morphology, cell division, and sensitivity for antibiotics, and demonstrated that the diversity of the *rodA* gene is sufficient to identify viridans streptococci at the species level. Moreover, we developed a more convenient denaturing gradient gel electrophoresis (DGGE) method based on the diversity of the *rodA* gene (*rodA*-DGGE) for detecting nine dominant streptococcal species in human saliva, namely, *Streptococcus sanguinis*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus parasanguinis*, *Streptococcus gordonii*, *Streptococcus vestibularis*, *Streptococcus salivarius*, *Streptococcus mutans,* and *Streptococcus sobrinus.*

Results: This *rodA*-DGGE method proved useful in detecting viridans streptococci without cultivation, isolation, and phenotypic characterization.

Conclusion: Analysis of the oral microbiota by *rodA*-DGGE offers a higher resolution than the conventional DGGE using 16S rDNA and may be an alternative in the microbial diagnosis of streptococcal infection.

Keywords: *population analysis*; *oral microbiota*; *streptococcus*; *subacute bacterial endocarditis*; *dental caries*; *saliva*

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Denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene (16S rDNA) is used for investigating entire bacterial communities without cultivation (1). The advantage of this method is that it uses 16S rDNA, which is present in all bacteria and can be amplified with a set of universal bacterial primers. Bacterial species are differentiated on the basis of differential migration on a denaturing gradient gel due to their melting behavior, which is based on the difference in the G+C content. A bar code-like profile is obtained, with each band presumably representing a different microorganism within the microbial communities (1–3). This molecular technique has become an important tool for studying complex bacterial communities and has been applied for the analyses of various microbiotas, such as those found in environmental biofilms, food fermentation processes, feces, intestine, gastrointestinal tract infections, corneal ulcer, and vaginosis (4–10).

DGGE analysis using 16S rDNA (16S rDNA-DGGE) has also been applied for analyzing the microflora found in the periodontal pocket, dental plaque, and saliva in order to identify the pathogens causing periodontitis, dental caries, and halitosis (11–14). These reports indicate

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that the pathogenic bacteria of oral diseases, such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Streptococcus mutans*, are clearly detected by 16S rDNA-DGGE. However, viridans streptococci, which are closely associated with bacteremia and subacute bacterial endocarditis (SBE), have not been clearly detected. One of the reasons is that the 16S rDNA genes of several viridans streptococci are highly homologous and that there is evidence of homologous recombination between species (15). The 16S rDNA genes of *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus pneumoniae*, in particular, exhibit more than 99% sequence homology with each other (16). Therefore, it is difficult to clearly identify these species by 16S rDNA-DGGE as this method is based on differences in the G+C content. Hence, our hypothesis was that some other gene, the evolutionary rate of which is higher than that of 16S rDNA, would be a useful target for DGGE analysis aiming at detecting the abovementioned streptococci.

Previously, during the sequencing of the glucosyltransferase gene (*gtfR*) of *S. oralis* (17), we found three open reading frames upstream of *gtfR*. By using the basic local alignment search tool, one of them was identified as the rod shape-determining protein (RodA) gene (*rodA*), which is widely present in gram-positive and gram-negative bacteria. This gene determines cellular morphology and is associated with peptidoglycan degradation during elongation and septation (18). In addition, since it has been suggested that *rodA* of *Streptococcus thermophilus* is associated with oxidative stress defense and streptonigrin tolerance (19, 20), it becomes one of the interesting genes in the study of bacterial response against environmental stress. Our preliminary alignment analysis revealed 78% similarity of *rodA* of *S. oralis* to that of *S. mitis*; further, this similarity was lower than that between 16S rDNA of *S. oralis* and that of *S. mitis*. Thus, we thought that the streptococcal *rodA* gene would exhibit sufficient phylogenetic diversity to aid in identifying viridans streptococci by DGGE.

In this study, we investigated the prevalence of the *rodA* genes in viridans streptococci, and phylogenetically analyzed them as the identification tool of those species. In addition, we developed a DGGE method based on the phylogenetic diversity of the *rodA* gene for detecting dominant streptococci in human oral cavity.

Methods

Sampling of streptococcal strains in saliva

The study group comprised eight healthy adult volunteers. The study protocol was approved by the Ethics Board of the Institute of Dentistry, Nagasaki University, and informed consent was obtained from all the subjects. Non-stimulated saliva was used as the clinical sample in this study, since it roughly represents a summary of the oral microbiota of the teeth, tongue, and mucosa of the upper respiratory tract. These samples were obtained by collecting whole saliva in a sterile tube before tooth brushing. The samples were serially diluted and inoculated on mitis-salivarius (MS) agar (Difco Laboratories, Detroit, MI). From the inoculated MS agar plates, 20 colonies were randomly selected and streptococcal strains in saliva were isolated. These clinical isolates were identified by a combination of phenotypic characterization performed using STREPTOGRAM (Wako Pure Chemicals, Osaka, Japan) (21), polymerase chain reaction (PCR) based on the species-specific variety of *gtf* genes (22), and sequencing of the 16S rRNA gene (16) and were examined for the *rodA* sequence according to the following method.

Bacterial strains and culture

The reference strains used in this study were taken from our own culture collection (Table 1) (21, 23). These were selected as the streptococcal species that could be detected in the oral cavity. The strains designated ATCC, NCTC, CCUG, and GTC were obtained from the American Type Culture Collection, National Collection of Type Cultures (Colindale, London, England), Culture Collection of the University of Göteborg (Göteborg, Sweden), and Gifu Type Culture Collection (Gifu, Japan), respectively. These organisms were routinely cultured in brain heart infusion broth (BHI; Difco Laboratories) and on 5% defibrinated sheep blood agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

Preparation of DNA for PCR

DNA of the cultured bacteria was obtained as previously described (17). In brief, the organisms were grown in BHI broth at 37°C for 18 h, collected, and then washed by centrifugation. The cells were suspended in a solution of 50 mM NaCl and 10 mM Tris-HCl (pH 7.4) and then digested with mutanolysin (final concentration, 33.3 U mL^{-1}; Sigma-Aldrich Co., St. Louis, MO) at 50°C for 1 h. Thereafter, the cells were lysed by adding *N*-lauroyl sarcosine (final concentration, 1.5%) and Ethylenediamin etetraacetic acid (EDTA) (final concentration, 10 mM). The lysate was treated with RNase (0.3 mg mL^{-1}; Wako Pure Chemicals) and proteinase K (0.3 mg mL^{-1}; Sigma-Aldrich Co.). DNA was purified from the cell lysate by phenol, phenol-chloroform, and chloroform extractions and collected by ethanol precipitation.

Further, the bacterial DNA from the saliva samples was extracted as previously described (22). In brief, the organisms in the saliva were harvested from 500 µL of the samples by centrifugation at 16,000 × *g* for 10 min. The bacterial cells were heated in a microwave oven at 500 W for 5 min to destroy the cell walls and then digested in 100 µL of 200 U mL^{-1} mutanolysin (Sigma-Aldrich Co.) at

Table 1. *Streptococcus* strains used in this study

Streptococcus group	Accession number		
Species	Strain	*rodA*	16S rDNA
Mitis group			
S. mitis	NCTC 12261^T	SMT1128[a]	D38482
S. pseudopneumoniae	CCUG 49455^T	AB441144[b]	AY485599
S. oralis	ATCC 10557	AB439009[b]	AB355617
S. gordonii	ATCC 10558^T	AB441145[b]	AY485606
S. sanguinis	ATCC 10556^T	AB441146[b]	DQ303192
S. parasanguniis	ATCC 15912^T	AB441147[b]	DQ303191
S. infantis	ATCC 27375^T	AB441148[b]	AB008315
S. australis	ATCC 700641^T	AB441149[b]	AY485604
S. cristatus	NCTC 12479^T	AB441150[b]	AB008313
S. peroris	GTC848^T	AB441151[b]	AB008314
Salivarius group			
S. salivarius	NCTC 8618^T	AB441152[b]	AB355616
S. vestibularis	ATCC 49125^T	AB441153[b]	AY188353
S. thermophilus	ATCC 19258^T	AB441154[b]	X68418
Anginosus group			
S. anginosus	ATCC 33397^T	AB441155[b]	AB355609
S. constellatus subsp. constellatus	ATCC 27823^T	AB441156[b]	AB355606
S. constellatus subsp. pharyngis	CCUG 46377^T	AB441157[b]	AY309095
S. intermedius	ATCC 27355^T	AB441158[b]	AF104671
Mutans group			
S. mutans	NCTC 10449^T	AB441159[b]	AB294730
S. sobrinus	ATCC 27351	AB441160[b]	AF439398
Bovis group			
S. equinus	ATCC 33317[c]	AB441161[b]	AB002482
S. gallolyticus subsp. macedonicus	CCUG 39970^T	AB441162[b]	AF459431

^{T}T means type strain.
[a]TIGR locus name in the genome database of TIGR.
[b]Accession number of a sequence determined in this study.
[c]This strain is the type strain of once '*Streptococcus bovis*.'

50°C for 1 h. The lysate was treated with 80 μL of nuclei lysis solution (Promega, Madison, WI) at 80°C for 5 min, and the proteins were removed by centrifugation after adding 60 μL of protein precipitation solution (Promega). The DNA was then purified by phenol-chloroform extraction and collected by ethanol precipitation.

Alignment analysis and construction of the phylogenetic tree

ClustalX software (24), downloaded from http://www.ebi.ac.uk, was used to align the sequences. Phylogenetic tree was constructed by using the neighbor-joining algorithm with MEGA 4 (25) on the basis of nucleotide sequences by using the maximum composite likelihood model. The corresponding parameter of the neighbor-joining algorithm was set as 'complete deletion.'

Phylogenetic analysis of the known rodA sequences

To evaluate the appropriateness of the phylogenetic reconstruction based on *rodA* genes and to design the primers used in this study, relevant, available gene sequences of *rodA* and 16S rDNA genes were obtained from the GenBank database and analyzed. The phylogenetic distances were calculated with the abovementioned algorithm and parameter using MEGA 4.

Design of PCR primers

The primers used in this study to amplify the fragments of the *rodA* gene and to determine their sequence were Rd_uni-F (5′-CCDTCAGARTTTATGAAGATWTCC-3′) and Rd_uni-R (5′-AATCATATCHSWYTCDCG-DACWGG-3′). Approximately 520-bp-long fragment of the *rodA* gene was amplified with these primers. These oligonucleotide primers were designed on the basis of conserved sequences, which were identified by aligning the streptococcal *rodA* genes in the GenBank nucleotide database. The DGGE sample was amplified using Rd_uni-F and a primer constructed by the addition of the GC clamp (CGCCCGGGGCGCGCCCCGGGC GGCCCGGGGGCACCGGGGG-) to Rd_uni-R.

PCR conditions

The *rodA* gene was amplified by performing PCR in 50 μL of a reaction mixture containing 0.5-U Takara Ex Taq™ Hot Start (HS) Version (Takara, Kyoto, Japan), 0.5 μM of the oligonucleotide primers, template DNA ($<$20 ng μL^{-1}), and 1.5 mM of $MgCl_2$, according to the manufacturer's instructions. Amplification was performed using GeneAmp® System 9700 (Applied Biosystems, Foster City, CA), under the following conditions: 35 cycles of denaturation at 98°C for 10 s, primer annealing at 48°C for 30 s, and extension at 72°C for 30 s. The PCR products were analyzed by 1.5% agarose gel electrophoresis, after staining with ethidium bromide. The amplicons were then purified with a Qiagen PCR purification kit (QIAGEN GmbH, Hilden, Germany) and used as the template for subsequent sequencing and amplification of the DGGE sample. The DGGE samples were amplified by PCR by changing the annealing temperature to 53°C and using the purified amplicons as the template.

Sequencing and analysis of the streptococcal rodA genes

The cycle sequencing reaction of the purified amplicons were performed by using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the products

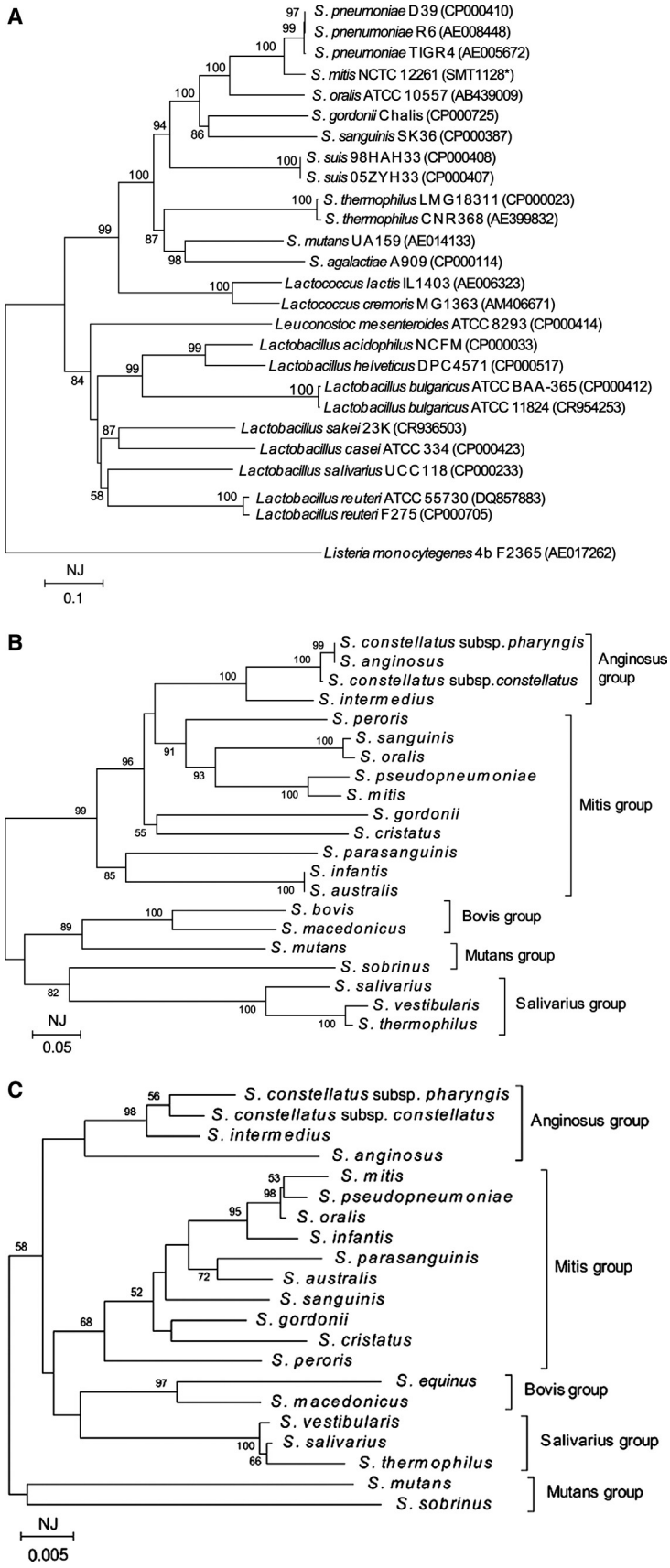

Fig. 1. (*Continued*)

were examined with an automatic DNA sequencer (ABI Prism 3,100; Applied Biosystems), according to the manufacturer's instructions. The resultant sequences were aligned using ClustalX and then analyzed with MEGA 4, as described above.

DGGE analysis

In this study, the DGGE analyses were performed with the DCode^{TM} universal mutation detection system (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. The DGGE analysis was initially performed with a perpendicular denaturing gradient gel in order to determine the conditions for that with a parallel gradient gel. To determine the streptococcal *rodA* genes having the highest and lowest melting temperature (T_m) among nine dominant streptococcal species in the human oral cavity, *S. mitis*, *Streptococcus sanguinis*, *S. oralis*, *Streptococcus gordonii*, *Streptococcus parasanguinis*, *Streptococcus salivarius*, *Streptococcus vestibularis*, *S. mutans,* and *Streptococcus sobrinus* (26, 27), the T_m values of the *rodA* amplicons were calculated using the following formula: $T_m = 81.5°C + 16.6\ (\log_{10}[Na^+]) + 0.41(\%[G+C]) - (500/n)$ (28). The samples with genes having the highest and lowest T_m values were applied on a perpendicular denaturing gradient gel containing 6% of acrylamide and 0–70% linear gradient of denaturant (100% denaturant was equivalent to 7 mol·L^{-1} urea and 40% deinonized formamide) and separated at 80 V for 2 h at 56°C. On the basis of the result obtained, the appropriate constant denaturant concentration in the DGGE analysis to detect the selected nine streptococcal species was manually determined by changing it into every 2% from the dissociable denaturant concentration of the lowest-T_m *rodA* fragment to that of the highest-T_m fragment. The electrophoresis patterns of nine streptococcal reference strains were adopted as the reference markers to identify these species. In the constant denaturing gel electrophoresis, a gel containing 8% (w/v) of acrylamide and 28% of denaturant was used, and electrophoresis was performed at 260 V for 6 h at 56°C. The electrophoresed gels were visualized by staining with SYBR® Gold (Invitrogen Corp., Carlsbad, CA).

Registration of sequences

The newly determined *rodA* sequences were deposited in DDBJ. Their accession numbers are shown in Table 1.

Results

Phylogenetic analysis of reference rodA genes from selected gram-positive bacteria and sequence analysis of streptococcal rodA genes

At first, in order to investigate the prevalence of *rodA* gene in *Streptococcus* spp., we performed phylogenetic analysis of the sequences obtained from members of the genus *Streptococcus* and related genera. We obtained 26 *rodA* genes, derived from the members of the genera *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Listeria*, from the GenBank database. The phylogenetic tree of these genes was constructed, with its root representing the *rodA* gene of *Listeria monocytogenes* (Fig. 1A). This tree clearly divides the tested strains at the genus and species levels. Therefore, the streptococcal *rodA* genes were considered to exhibit sufficient phylogenetic diversity to classify the genus at the species level. Moreover, when the sequence similarities of *rodA* genes among *S. pneumoniae* R6, *S. mitis* NCTC 12261, and *S. oralis* ATCC 10557 were calculated, the similarity between *S. pneumoniae* and *S. mitis*, *S. pneumoniae* and *S. oralis*, and *S. oralis* and *S. mitis* was 93%, 77%, and 78%, respectively. These similarity values were smaller than those of the 16S rRNA genes among the three abovementioned strains.

Next, we performed the alignment analysis of the streptococcal *rodA* gene sequences retrieved in order to design the universal PCR primers that could amplify all the streptococcal *rodA* genes. As a result, we constructed the primers that could amplify approximately 520-bp-long fragment from the N-terminus region of 1,200-bp-total length of the *rodA* gene and the amplicons isolated from 21 nonhemolytic streptococci were sequenced (Table 1). In the phylogenetic analysis, approximately 370-bp sequence determined with fidelity was used.

Phylogenetic analysis of the streptococcal rodA genes

The phylogenetic distances of the *rodA* (approximately 370 bp) and 16S rDNA (approximately 1,200 bp) genes among streptococcal strains in Table 1 were computed with MEGA 4 by using the following: bootstrap analyses (500 replicates), gap/missing data (complete deletion), model (maximum composite likelihood), and substitution (d; transition + transversion) (Table 2). The mean phylogenetic distance of the *rodA* and 16S rDNA genes was calculated and found to be 0.527 (SE, 0.474) and 0.043 (SE, 0.005), respectively. For this reason, the base

Fig. 1. Phylogenetic analyses of the sequences of the *rodA* gene. A, Phylogenetic tree derived from gram-positive bacteria's *rodA* gene, which was obtained from the GenBank database. The numbers in parentheses indicate the GenBank accession numbers. Comparison of the phylogenetic trees of partial sequences of *rodA* and 16S rDNA constructed using the reference strains of nonhemolytic streptococci in Table 1. B, Phylogenetic tree derived from partial sequences of *rodA* (370 bp) and C, Phylogenetic tree derived from partial sequences of 16S rDNA (1,277 bp). The scales of both phylogenetic trees were adjusted so that they were equivalent.

Table 2. Comparison of phylogenetic distance between *rodA* (lower triangle) and 16S rDNA (upper triangle) of type strains of *Streptococcus* species

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]
[1] *S.mitis*		0.01	0.00	0.03	0.03	0.03	0.01	0.02	0.03	0.03	0.05	0.05	0.06	0.06	0.04	0.04	0.04	0.07	0.06	0.06	0.05
[2] *S. pseudopneumoniae*	0.08		0.00	0.03	0.02	0.02	0.01	0.02	0.03	0.02	0.05	0.05	0.06	0.06	0.04	0.04	0.04	0.06	0.06	0.06	0.05
[3] *S. oralis*	0.29	0.28		0.02	0.02	0.02	0.01	0.02	0.03	0.02	0.05	0.05	0.05	0.06	0.04	0.04	0.04	0.06	0.06	0.06	0.05
[4] *S. gordonii*	0.39	0.42	0.46		0.02	0.03	0.02	0.02	0.02	0.03	0.04	0.04	0.05	0.05	0.03	0.04	0.03	0.05	0.05	0.05	0.04
[5] *S. sanguinis*	0.27	0.27	0.02	0.46		0.03	0.02	0.02	0.03	0.03	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.05	0.06	0.05	0.04
[6] *S. parasanguinis*	0.46	0.45	0.52	0.52	0.51		0.03	0.02	0.02	0.04	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.06	0.07	0.06	0.04
[7] *S. infantis*	0.40	0.42	0.48	0.53	0.47	0.39		0.02	0.03	0.03	0.04	0.04	0.05	0.06	0.04	0.04	0.04	0.06	0.06	0.05	0.04
[8] *S. australis*	0.40	0.42	0.48	0.53	0.47	0.39	0.00		0.02	0.03	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.06	0.06	0.05	0.05
[9] *S. cristatus*	0.45	0.49	0.41	0.42	0.42	0.48	0.50	0.50		0.04	0.04	0.05	0.05	0.05	0.04	0.05	0.04	0.06	0.07	0.06	0.04
[10] *S. peroris*	0.31	0.32	0.32	0.43	0.31	0.47	0.48	0.48	0.41		0.03	0.03	0.04	0.05	0.04	0.05	0.04	0.06	0.07	0.05	0.04
[11] *S. salivarius*	0.65	0.71	0.71	0.73	0.70	0.65	0.60	0.60	0.74	0.64		0.00	0.01	0.05	0.05	0.06	0.04	0.06	0.07	0.05	0.04
[12] *S. vestibularis*	0.75	0.79	0.76	0.79	0.79	0.67	0.62	0.62	0.79	0.67	0.17		0.01	0.04	0.05	0.06	0.04	0.06	0.06	0.05	0.04
[13] *S. thermophilus*	0.72	0.75	0.75	0.78	0.79	0.66	0.61	0.61	0.78	0.64	0.16	0.03		0.05	0.05	0.06	0.05	0.06	0.07	0.05	0.04
[14] *S. anginosus*	0.38	0.38	0.41	0.46	0.41	0.47	0.45	0.45	0.40	0.35	0.72	0.73	0.70		0.04	0.04	0.03	0.06	0.06	0.05	0.04
[15] *S. constellatus subsp. constellatus*	0.38	0.38	0.40	0.44	0.39	0.46	0.44	0.44	0.38	0.34	0.70	0.72	0.69	0.02		0.01	0.01	0.06	0.05	0.05	0.04
[16] *S. constellatus subsp. pharyngis*	0.38	0.38	0.41	0.46	0.41	0.47	0.45	0.45	0.4	0.35	0.72	0.73	0.70	0.00	0.02		0.02	0.07	0.06	0.06	0.05
[17] *S. intermedius*	0.40	0.38	0.39	0.39	0.37	0.45	0.45	0.45	0.38	0.33	0.66	0.72	0.69	0.16	0.15	0.16		0.06	0.05	0.06	0.05
[18] *S. mutans*	0.59	0.64	0.67	0.62	0.67	0.60	0.57	0.57	0.61	0.6	0.53	0.57	0.55	0.66	0.64	0.66	0.62		0.07	0.06	0.05
[19] *S. sobrinus*	0.68	0.72	0.70	0.67	0.67	0.70	0.64	0.64	0.69	0.65	0.52	0.61	0.61	0.73	0.74	0.73	0.64	0.54		0.06	0.05
[20] *S. bovis*	0.62	0.66	0.64	0.64	0.67	0.66	0.62	0.62	0.63	0.66	0.62	0.62	0.60	0.63	0.61	0.63	0.60	0.38	0.70		0.03
[21] *S. gallolyticus subsp. macedonicus*	0.63	0.63	0.66	0.62	0.66	0.61	0.67	0.67	0.59	0.63	0.64	0.62	0.63	0.60	0.59	0.60	0.62	0.41	0.59	0.23	

Note: Each number written from right to left corresponds to the same-numbered bacterial species from the top to the bottom.

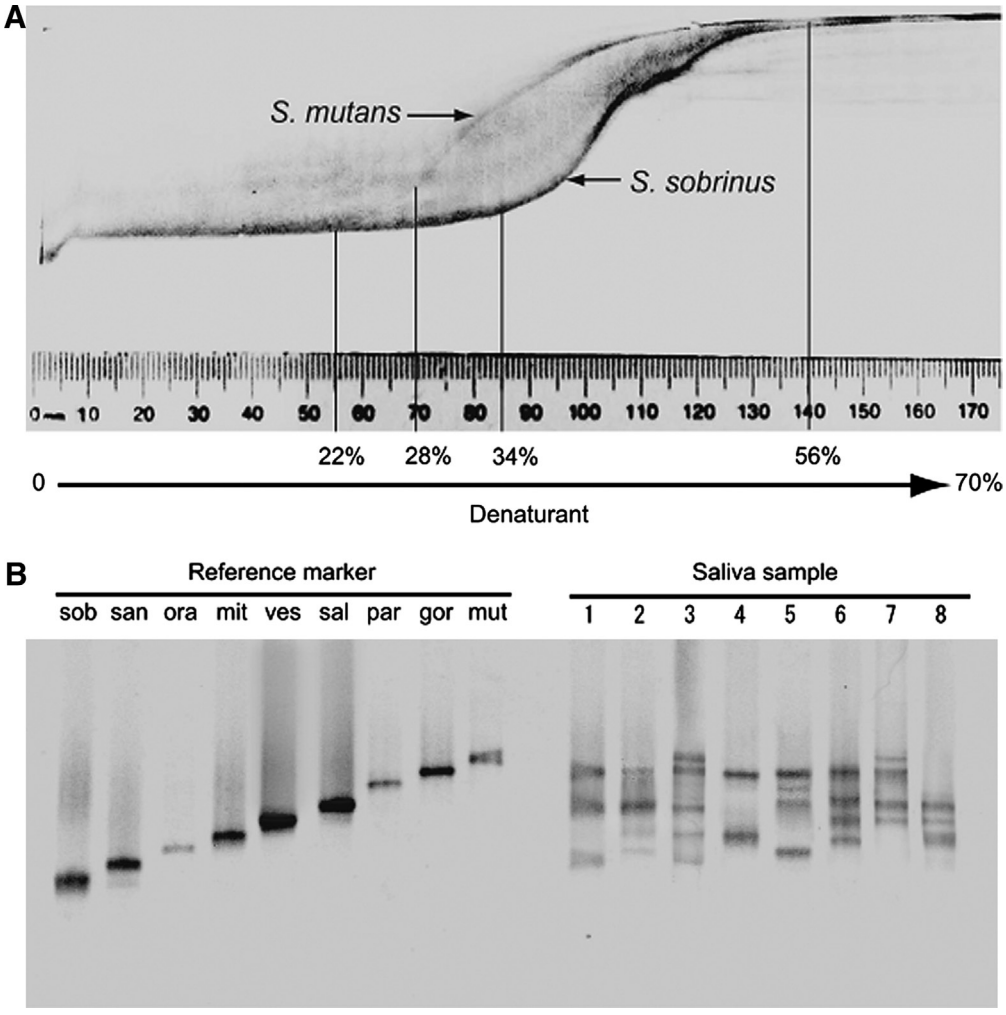

Fig. 2. Negative image of the *rodA*-DGGE analyses. The appropriate denaturant concentration in the following experiment was determined by the ethidium bromide-stained perpendicular DGGE gel. The *rodA* gene fragments of *S. sobrinus* (with highest T_m) and *S. mutans* (with lowest T_m) were amplified with the GC-clamped primer set. The amplicons were applied on the same perpendicular denaturing gradient gel containing 6% of acrylamide and 0–70% linear gradient of denaturant and electrophoresed at 80 V for 2 h at 56°C. The electrophoretic bands of these two amplicons were separated at concentrations of the denaturant ranging from 22 to 56% (A). The *rodA*-DGGE analysis with parallel constant denaturant gel was applied to detection of viridans streptococci in saliva. In this experiment, a parallel constant denaturant gel containing 8% (w/v) of acrylamide and 28% of constant denaturant was used, and the GC-clamped *rodA* gene fragments were separated at 260 V for 6 h at 56°C in 0.5 × Tris-acetate-EDTA buffer. As the reference markers to identify the streptococcal species, the GC-clamped *rodA* fragments of *S. sobrinus* (sob), *S. sanguinis* (san), *S. oralis* (ora), *S. mitis* (mit), *S. vestibularis* (ves), *S. salivarius* (sal), *S. parasanguinis* (par), *S. gordonii* (gor), and *S. mutans* (mut) were used. The Arabic numerals identify the individual subjects (B).

substitution rates in the *rodA* genes were observed to be considerably higher than those in the 16S rDNA sequences, though the phylogenetic distance between the *rodA* genes of *Streptococcus australis* and *Streptococcus infantis* was 0.00. The phylogenetic tree also indicated that the evolutionary rates of the *rodA* genes, except those of *S. australis* and *S. infantis*, were higher than those of the 16S rDNA sequences (Fig. 1B and C). Thus, it was revealed that the phylogenetic analysis based on the *rodA* sequences of *Streptococcus* spp. except *S. australis* and *S. infantis*, would be able to differentiate the species that are closely related by 16S rDNA analysis.

Determination of the conditions for DGGE with constant denaturing gradient gel

As for the nine dominant streptococcal species in the human oral cavity, the G+C content (%) and T_m value (°C) were estimated from these sequence data in order to determine the conditions for the subsequent DGGE analysis. The *rodA* genes of *S. sobrinus* and *S. mutans* showed the highest (49.7°C) and lowest (37.6°C) T_m values, respectively. The fragments of the genes obtained from these two strains, which were amplified with the GC-clamped primer set, were applied to perpendicular denaturing gradient gel to determine the optimal concentration

of the denaturant (Fig. 2A). It was observed that these two amplicons separated between 22% and 56% of the denaturant and that the double-strand *rodA* fragment of *S. mutans* and *S. sobrinus* started to denature at 22% and 34% of the denaturant, respectively. Thus, the appropriate constant denaturant concentration in the DGGE analysis to detect the nine streptococcal species was fixed at 28%. In this condition, the *rodA* fragments from the nine species showed clearly different mobility (Fig. 2B) in order of the estimated T_m value except *S. sanguinis* and *S. mitis* (data not shown).

Detection of nine Streptococcus species by DGGE analysis based on the diversity of the rodA gene

As shown in Fig. 2B, the electrophoresis patterns of the *rodA* gene fragments of the clinical samples were compared with those of nine reference streptococcal strains, and the streptococci present in the saliva samples were expected (Table 3). Then, we extracted the DNA fragments from the band on the DGGE gel in Fig. 2B and carried out direct sequence. The sequence of the streptococci asterisked in Table 3 were consistent with the *rodA* sequence of the corresponding bacterial species isolated from MS agar, although the fragments extracted from thin bands in lanes 2, 3, and 4 could not be sequenced. On the other hand, all species listed in Table 3 were contained in the sample of streptococci isolated from MS agar plate cultures. Thus, it was revealed that the *rodA*-DGGE analysis could detect the streptococcal species as well as the cultivation method.

Discussion

RodA is the molecule that participates with penicillin-binding protein 2 in peptidoglycan synthesis and cell division (29). Peptidoglycan synthesis by these two molecules has been investigated in association with susceptibility to antibiotics such as penicillin (30), and many studies on this subject have been reported not only on gram-negative bacilli such as *Escherichia coli* or *Salmonella* (31–33) but also on gram-positive cocci such as *S. pneumoniae* and viridans streptococci (34–41). Therefore, it is important that peptidoglycan synthesis and cell division by RodA and penicillin-binding protein 2 is studied in these streptococci in the future. Further, it is thought that phylogenetic analysis of the streptococcal *rodA* gene, which is one of the genes associated with the abovementioned biological activities, is more important as the initial step in the investigation of the mechanism underlying drug resistance in streptococcal infection. However, until now, there is no report on the phylogenetic analysis of the streptococcal *rodA* gene. In the present study, we investigated the prevalence of the *rodA* gene in viridans streptococci and determined the phylogenetic relationship of the streptococcal *rodA* gene in certain representative gram-positive bacteria by constructing a phylogenetic tree. It was revealed that the genus *Streptococcus*, together with the genus *Lactococcus*, formed one cluster of cocci in the dendrogram rooted by genus *Listeria*, while *Leuconostoc mesenteroides* was classified in the bacilli cluster.

In the field of the water examination, since the microbial population associated with denitrification cannot be precisely identified by 16S rDNA-DGGE alone, DGGE methods based on *nirS* and *nirK* have been developed and applied (42–44). Similarly, the application of the DGGE to analysis of other housekeeping and/or prevalent genes as an alternative or supplement to 16S rDNA-DGGE is thought to offer a higher resolution to analyses of complex microbial populations such as that of the human oral cavity. In this study, for developing the DGGE method for the analysis of the oral streptococcal population, we used the *rodA* gene. We first performed a phylogenetic analysis of the *rodA* gene derived from the 21 streptococcal species that may be isolated from the oral cavity. The analysis results revealed that the phylogenetic tree of *rodA* classified these streptococci, except *S. australis* and *S. infantis*, with a phylogenetic resolution that was 10 times that of the phylogenetic tree based on 16S rDNA. These results suggested that the *rodA* gene possesses sufficient genetic diversity to identify viridans streptococci and that phylogenetic analysis of this gene may be an efficient tool for their classification and identification. However, this phylogenetic analysis has a potential limitation, since any analysis of oral streptococci based on single gene loci may be flawed by inter-species homologous recombination, which is not uncommon in these bacteria (15, 45). In the present study, one possible example of this may be the lack of discrimination between *S. australis* and *S. infantis*. For this reason, we should make an allowance for homologous recombination in the identification based on single gene loci.

Next, we succeeded in developing a DGGE method based on the diversity of *rodA* (*rodA*-DGGE) in order to

Table 3. The *rodA*-DGGE analysis of clinical samples

No.	Detected streptococci
1	*S. gordonii*, S. salivarius*, S. oralis**
2	*S. gordonii*, S. salivarius*, S. mitis, S. oralis*
3	*S. mutans*, S. gordonii*, S. parasanguinis, S. salivarius*, S. mitis*, S. sanguinis**
4	*S. gordonii*, S. salivarius, S. mitis**
5	*S. gordonii*, S. parasanguinis*, S. salivarius*, S. oralis**
6	*S. gordonii*, S. salivarius*, S. vestibularis*, S. mitis**
7	*S. mutans*, S. gordonii*, S. salivarius*, S. vestiburalis**
8	*S. salivarius*, S. vestibularis*, S. mits*, S. oralis*

*The asterisked streptococci were consistent with the *rodA* sequence of the corresponding bacterial species.

detect nine selected streptococcal species, which frequently are isolated from the oral cavity and associated with oral disease such as dental caries, and systemic disease such as bacteremia and SBE (21). As we could detect and differentiate the nine species in saliva by *rodA*-DGGE without cultivation, it was suggested that our developed method is an efficient initial screening test for the detection of the pathogenic and commensal streptococci derived from the human oral cavity. Especially, in the situation where the SBE-causing streptococci had been isolated, our *rodA*-DGGE would provide the opportunity to simultaneously evaluate the existence of the infecting organism in the oral cavity, blood, the infected organ, and saliva of the patient. Even though a causative organism cannot be identified by this DGGE method, sequencing analysis followed by Basic Local Alignment Search Tool (BLAST) search of the tested *rodA* fragment will be able to identify the species among the other 12 *Streptococcus* species that were not adopted as reference markers. Although these 12 *Streptococcus* species are minor in human saliva, e.g. *Streptococcus anginosus* is one of the bacteria associated with bacteremia (21). For this reason, a database of *rodA* gene sequences as well as other housekeeping genes is important (45). Moreover, we believe that identification based on a combination of phylogenetic analysis of *rodA* gene and 16S rDNA offers a higher resolution and overcomes weaknesses in each method. For example, although our method could not distinguish *S. infantis* and *S. australis*, the phylogenetic analysis of 16S rDNA could classify these species (46, 47). On the other hand, although the phylogenetic analysis of 16S rDNA could not clearly identify *S. mitis*, *S. oralis*, and *S. pneumoniae* (16), our method could divide them.

In conclusion, we showed the prevalence and phylogenetic analysis of *rodA* gene in viridans streptococci and demonstrated that identification based on the diversity of *rodA* genes, containing *rodA*-DGGE, was convenient and effective as an initial screening of viridans streptococci. In our future clinical study, we will apply the phylogenetic analysis of *rodA* gene containing *rodA*-DGGE to the detection of SBE-causative streptococci by using the bacterial DNA samples extracted from the isolated streptococci, the blood, infected organ, and saliva of their host.

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***T. Hoshino**
Department of Pediatric Dentistry
Nagasaki University Graduate School of Biomedical Sciences
1-7-1 Sakamoto
Nagasaki 852-8588, Japan
Tel: +81-95-819-7674
Fax: +81-95-819-7675
Email: thoshino@nagasaki-u.ac.jp