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

Ikuri Konishi¹, Tomonori Hoshino¹*, Yoshio Kondo¹,², Kan Saito¹, Miyuki Nishiguchi¹, Kyoko Sato¹ and Taku Fujiwara¹

¹Department of Pediatric Dentistry, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; ²Division 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 gordoni*, *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

Received: 9 June 2009; Revised: 29 July 2009; Accepted: 31 July 2009; Published: 28 August 2009

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 vaginitis (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
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 streptocinigrin 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 Ethylenediaminetetraacetic 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 | Strain         | Accession number | 16S rDNA |
|-------------------|---------------|------------------|---------|
| Mitis group       | NCTC 12261<sup>T</sup> | SMT1128<sup>a</sup> | D38482  |
|                   | CCGU 49455<sup>T</sup> | AB441144<sup>b</sup> | AY485599 |
| S. mitis          | ATCC 10557    | AB439009<sup>b</sup> | AB355617 |
| S. gordonii       | ATCC 10558<sup>T</sup> | AB441145<sup>b</sup> | AY485606 |
| S. sanguinis      | ATCC 15912<sup>T</sup> | AB441147<sup>b</sup> | D303191  |
| S. infantis       | ATCC 27375<sup>T</sup> | AB441148<sup>b</sup> | AB008315 |
| S. australis      | ATCC 70064<sup>T</sup> | AB441149<sup>b</sup> | AY485604 |
| S. cristatus      | NCTC 12479<sup>T</sup> | AB441150<sup>b</sup> | AB008313 |
| S. salivarius     | GTCC848<sup>b</sup> | AB441151<sup>b</sup> | AB008314 |
| Salivarius group  | NCTC 8618<sup>T</sup> | AB441152<sup>b</sup> | AB355616 |
| S. vestibularis   | ATCC 49125<sup>T</sup> | AB441153<sup>b</sup> | AY188353 |
| S. thermophilus   | ATCC 19258<sup>T</sup> | AB441154<sup>b</sup> | X68418  |
| Anginosis group   | ATCC 33397<sup>T</sup> | AB441155<sup>b</sup> | AB355609 |
| S. anginosus      | ATCC 27823<sup>T</sup> | AB441156<sup>b</sup> | AB355606 |
| subsp. constellatus | CCGU 46377<sup>T</sup> | AB441157<sup>b</sup> | AY309095 |
| subsp. pharyngis  | ATCC 27355<sup>T</sup> | AB441158<sup>b</sup> | AF104671 |
| Mutans group      | NCTC 10449<sup>T</sup> | AB441159<sup>b</sup> | AB294730 |
| S. mutans         | ATCC 27351    | AB441160<sup>b</sup> | AF439398 |
| S. sobrinus       | ATCC 10557    | AB441161<sup>b</sup> | AB002482 |
| S. equinus        | ATCC 33317<sup>C</sup> | AB441162<sup>b</sup> | AF459431 |
| subsp. macedonicus| CCGU 39970<sup>T</sup> | AB441163<sup>b</sup> | AF459431 |

1<sup>T</sup> means type strain.
2<sup>a</sup>TIGR locus name in the genome database of TIGR.
3<sup>b</sup>Accession number of a sequence determined in this study.
4<sup>c</sup>This strain is the type strain of once 'Streptococcus bovis."

50°C for 1 h. The lysate was treated with 80 μL of nuclei
sible 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 phyloge-
netic 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'-CCGCGAGATTTATGAGATWCC-
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 (CGCCCGGGGGCCGCCGGGC
GGGCCCAGGAGGCACCAGAGG-) 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<sup>-1</sup>), and 1.5 mM of MgCl<sub>2</sub>, according to
the manufacturer’s instructions. Amplification was per-
formed using GeneAmp® System 9700 (Applied Biosys-
tems, 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 electrophor-
esis, 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™ 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: \[ T_m = 81.5^\circ C + 16.6 \log_{10}(\text{Na}^+) + 0.41(\% \text{G+C}) - (500/n) \] (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% deionized 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 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
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| 0.02| 0.02| 0.02| 0.02| 0.05| 0.05| 0.05| 0.06| 0.06| 0.04| 0.04| 0.06| 0.06| 0.06| 0.05|
| [4] S. gordonii | 0.39| 0.42| 0.46| 0.03| 0.02| 0.02| 0.02| 0.03| 0.02| 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| 0.05| 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| 0.43| 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| 0.05| 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| 0.05| 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| 0.04|
| [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| 0.04|
| [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| 0.04|
| [16] S. constellatus subsp. pharyngis | 0.38| 0.38| 0.41| 0.46| 0.41| 0.47| 0.45| 0.45| 0.45| 0.35| 0.72| 0.73| 0.70| 0.00| 0.02| 0.02| 0.07| 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| 0.05|
| [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|
| [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|
| [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| 0.60| 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.
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 Tm 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) Tm 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.

Fig. 2. Negative image of the rodA-DGGE analyses. The appropriate denaturant concentration in the following experiment was determined by the ethidium bromide-DGGE gel. The rodA gene fragments of S. sobrinus (with highest Tm) and S. mutans (with lowest Tm) 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).
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 Tm 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 in 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.

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. vestibularis* |
| 8   | S. salivarius*, S. vestibularis*, S. mitis*, 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.

Acknowledgements

This work was supported by grants-in-aid from the Japan Society for the Promotion of Science (18592243).

Conflict of interest and funding

This study was supported by Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. There is no conflict of interest in the present study for any of the authors.

References

1. Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993; 59: 695-700.
2. Bonin P, Michotey V, Mouzdhari A, Rontani J-F. Anaerobic biodegradation of squalene: using DGGE to monitor the isolation of denitrifying bacteria taken from enrichment cultures. FEMS Microbial Ecol 2002; 42: 37-49.
3. Muyzer G. DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol 1999; 2: 317-22.
4. Anukam KC, Reid G. Organisms associated with bacterial vaginosis in Nigerian women as determined by PCR-DGGE and 16S rRNA gene sequence. Afr Health Sci 2007; 7: 68-72.
5. Helig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD, de Vos WM. Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 2002; 68: 114-23.
6. Peixoto RS, da Costa Coutinho HL, Rumnajek NG, Macrae A, Rosado AS. Use of rpoB and 16S rDNA genes to analyse bacterial diversity of a tropical soil using PCR and DGGE. Lett Appl Microbiol 2002; 35: 186-20.
7. Randazzo CL, Pitino I, De Luca S, Sciò GO, Caggia C. Effect of wild strains used as starter cultures and adjunct cultures on the volatile compounds of the Pecorino Siciliano cheese. Int J Food Microbiol 2008; 122: 269–78.
8. Schabereiter-Gurtner C, Maca S, Kamiński S, Rolleke S, Lubitz W, Barisani-Asenbauer T. Investigation of an anaerobic microbrial community associated with a corneal ulcer by denaturing gradient gel electrophoresis and 16S rDNA sequence analysis. Diagn Microbiol Infect Dis 2002; 43: 193–9.
9. Songjinda P, Nakajima Y, Kuroki Y, Tanaka S, Fukuda S, Kiyohara C. Molecular monitoring of the developmental bacterial community in the gastrointestinal tract of Japanese infants. Biosci Biotechnol Biochem 2005; 69: 638–41.
10. Zoetendal EG, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rDNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 1998; 64: 3854-9.
11. Burton JP, Chilcott CN, Moore CJ, Speiser G, Tagg JR. A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. J Appl Microbiol 2006; 100: 754-64.
12. Li Y, Ku CY, Xu J, Saxena D, Caufield PW. Survey of oral microbiota diversity using PCR-based denaturing gradient gel electrophoresis. J Dent Res 2005; 84: 559-64.
13. Zijinge V, Harmsen HJ, Kleinfelder JW, van der Rest ME, Degener JE, Welling GW. Denaturing gradient gel electrophoresis analysis to study bacterial community structure in pockets of periodontitis patients. Oral Microbiol Immunol 2003; 18: 59–65.
14. Zijinge V, Welling GW, Degener JE, van Winkelhoff AJ, Abbas F, Harmsen HJ. Denaturing gradient gel electrophoresis as a diagnostic tool in periodontal microbiology. J Clin Microbiol 2006; 44: 3628–33.
15. Kilian M, Pouslen K, Blomqvist T, Håvarstein LS, Bek-Thomsen M, Tettelin H, et al. Evolution of *Streptococcus pneumoniae* and its close commensal relatives. PLoS ONE 2008; 16: e2683.
16. Kawamura Y, Hou XG, Sultana F, Miura H, Ezaki T. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordoni* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol 1995; 45: 406-8.
17. Fujiwara T, Hoshino T, Ooshima T, Sobue S, Hamada S. Purification, characterization, and molecular analysis of the gene encoding glucosyltransferase from Streptococcus oralis. Infect Immun 2000; 68: 2475-83.

18. Uehara T, Park JT. Growth of Escherichia coli: significance of peptidoglycan degradation during elongation and septation. J Bacteriol 2008; 190: 3914–22.

19. Thibessard A, Fernandez A, Gintz B, Leblond-Bourget N, Decaris B. Effects of rodA and pbp2b disruption on cell morphology and oxidative stress response of Streptococcus thermophilus CNRZ368. J Bacteriol 2002; 184: 2821-6.

20. Thibessard A, Borges F, Fernandez A, Gintz B, Decaris B, Leblond-Bourget N. Identification of Streptococcus thermophilus CNRZ368 genes involved in defense against superoxide stress. Appl Environ Microbiol 2004; 70: 2220-9.

21. Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J Clin Microbiol 2005; 43: 6073–5.

22. Hoshino T, Kawaguchi M, Shimizu N, Hoshino N, Ooshima T, Fujiwara T. PCR detection and identification of oral streptococci in saliva samples using gtf genes. Diagn Microbiol Infect Dis 2004; 48: 195–9.

23. Kilian M, Mikkelsen L, Henrichsen J. Taxonomic studies of viridans streptococci: description of Streptococcus gordonii sp. nov. and emended descriptions of Streptococcus sanguis (White and Niven 1946), Streptococcus oralis (Bridge and Sneath 1982), and Streptococcus mitis (Andrewes and Horder 1906). Int J Syst Bacteriol 1989; 48: 921–7.

24. Jeannougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trends Biochem Sci 1998; 23: 403–5.

25. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24: 1596–9.

26. Bek-Thomsen M, Tettelin H, Hance I, Nelson KE, Kilian M. Peptidoglycan synthetic activities in membranes of Streptococcus oralis, Streptococcus mitis, and Streptococcus infantis in the upper respiratory tracts of adults, determined by a nonculture strategy. Infect Immun 2008; 76: 3689–96.

27. Kononen E, Jousimies-Somer H, Bryk A, Kiku T, Kilian M. Establishment of streptococci in the upper respiratory tract: longitudinal changes in the mouth and nasopharynx up to 2 years of age. Med Microbiol 2002; 51: 723-30.

28. O’Gorman J, Russell D. Molecular cloning. Laboratory manual, third ed. New York: Cold Spring Harbor Laboratory Press: 2001.

29. Ishino F, Park W, Tomioka S, Tamaki S, Takase I, Kunugita K. Peptidoglycan synthetic activities in membranes of Escherichia coli caused by overproduction of penicillin-binding protein 2 and rodA protein. J Biol Chem 1986; 261: 7024–31.

30. Bylund JE, Haines MA, Walsh K, Boulo P, D’Ari R, Higgins ML. Buoyant density studies of several mecillinam-resistant and division mutants of Escherichia coli. J Bacteriol 1997; 179: 5396-402.

31. Costa CS, Anton DN. Round-cell mutants of Salmonella typhimurium produced by transposition mutagenesis: lethality of rodA and mre mutations. Mol Gen Genet 1993; 236: 387–94.

32. de Pedro MA, Donachie WD, Holte J, Schwar Z, Consta-tive septal murein synthesis in Escherichia coli with impaired activity of the mur genes. Proc Natl Acad Sci U S A 1990; 87: 5858–62.

33. Iwai N, Nagai K, Wach M. Novel S-benzylisothiourea compound that induces spherical cells in Escherichia coli probably by acting on a rod-shape-determining protein(s) other than penicillin-binding protein 2. Biosci Biotechnol Biochem 2002; 66: 2658–62.

34. Bilavsky E, Eliahou R, Keller N, Yarden-Bilavsky H, Harel L, Amir J. Effect of benzathine penicillin treatment on antibiotic susceptibility of viridans streptococci in oral flora of patients receiving secondary prophylaxis after rheumatic fever. J Infect 2008; 56: 244–8.

35. Doern GV, Richter SS, Miller A, Miller N, Rice C, Hellmann K. Antimicrobial resistance among Streptococcus pneumoniae in the United States: have we begun to turn the corner on resistance to certain antimicrobial classes? Clin Infect Dis 2005; 41: 139–48.

36. Dowson CG, Hutchison A, Woodford N, Johnson AP, George RC, Spratt BG. Penicillin-resistant viridans streptococci have obtained altered penicillin-binding protein genes from penicillin-resistant strains of Streptococcus pneumoniae. Proc Natl Acad Sci U S A 1990; 87: 5858–62.

37. Fujitani S, Rowlinson MC, George WL. Penicillin G-resistant viridans group streptococcal endocarditis and interpretation of the American Heart Association’s Guidelines for the Treatment of Infective Endocarditis. Clin Infect Dis 2008; 46: 1064–6.

38. Richter SS, Hellmann KP, Coffman SL, Huysh HK, Bruegge- mann AB, Pfäffler MA. The molecular epidemiology of penicillin-resistant Streptococcus pneumoniae in the United States, 1994-2000. Clin Infect Dis 2002; 34: 330–9.

39. Sevillano D, Aguilar L, Alou L, Gimenez MJ, Gonzalez N, Torrico M. Beta-lactam activity against penicillin-resistant Streptococcus pneumoniae strains exhibiting higher amoxicillin versus penicillin minimum inhibitory concentration values: an in vitro pharmacodynamic simulation. Chemotherapy 2008; 54: 84–90.

40. Westling K, Julander I, Ljungman P, Heimdahl A, Thalme A, Nord CE. Reduced susceptibility to penicillin of viridans group streptococci in the oral cavity of patients with haematological disease. Clin Microbiol Infect 2004; 10: 899–903.

41. Westling K, Julander I, Ljungman P, Jalal S, Nord CE, Wretlind B. Viridans group streptococci in blood culture isolates in a Swedish university hospital: antibiotic susceptibility and identification of erythromycin resistance genes. Int J Antimicrob Agents 2006; 28: 292–6.

42. Desnues C, Michotey VD, Wieland A, Zhizhong C, Fourcans A, Duran R. Seasonal and diel distributions of denitrifying and bacterial communities in a hypersaline microbial mat (Camargue, France). Water Res 2007; 41: 3407–19.

43. Goregues CM, Michotey VD, Bonin PC. Molecular, biochemical, and physiological approaches for understanding the ecology of denitrification. Micro Ecol 2005; 49: 198–208.

44. Shoji T, Nittami T, Onuki M, Satoh H, Mino T. Microbial community of biological phosphorus removal process fed with municipal wastewater under different electron acceptor conditions. Water Sci Technol 2006; 54: 81–9.

45. Bishop CJ, Aanensen DM, Jordan GE, Kilian M, Hanage WP, Spratt BG. Electronic taxonomy: assigning strains to bacterial species via the internet. BMC Biol 2009; 7: 3. doi:10.1186/1741-7007-7-3

46. Kawamura Y, Hou XG, Todome Y, Sultana F, Hirose K, Shu SE, Ezaki T, Ohkuni H. Streptococcus peroris sp. nov. and Streptococcus infantis sp. nov., new members of the Streptococcus mitis group, isolated from human clinical specimens. Int J Syst Bacteriol 1998; 48: 921–7.

47. Willcox MD, Zhu H, Knox KW. Streptococcus australis sp. nov., a novel oral streptococcus. Int J Syst Evol Microbiol 2001; 51: 1277–81.

* 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