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Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities

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SUMMARY

More than 700 bacterial species have been detected in the human oral cavity. They form highly organized microbial communities and are responsible for many oral infectious diseases, such as dental caries and periodontal disease. The prevention and treatment of these diseases require a comprehensive knowledge of oral microbial communities, which largely relies on culture-dependent methods to provide detailed phenotypic and physiological analysis of these communities. However, most of the currently available laboratory media can only selectively support the growth of a limited number of bacterial species within these communities, and fail to sustain the original oral microbial diversity. In this study, using denaturing gradient gel electrophoresis (DGGE) as an index to systematically survey and analyse the selectivity of commonly used laboratory media, we developed a new medium (SHI medium) by combining the ingredients of several selected media that can support different subpopulations within the original oral microbial community derived from pooled saliva. DGGE and 454 pyrosequencing analysis showed that SHI medium was capable of supporting a more diversified community with a microbial profile closer to that of the original oral microbiota. Furthermore, 454 pyrosequencing revealed that SHI medium supported the growth of many oral species that have not before been cultured. Crystal violet assay and the confocal laser scanning microscope analysis indicated that, compared with other media, SHI medium is able to support a more complex saliva-derived biofilm with higher biomass yield and more diverse species. This DGGE-guided method could also be used to develop novel media for other complex microbial communities.

INTRODUCTION

The oral microbiota is one of the most complex bacterial communities associated with the human body. More than 700 different bacterial species have been identified from the human oral cavity (Paster *et al.*, 2001, 2006; Aas *et al.*, 2005) and most are associated with dental plaque, forming highly organized microbial communities (Marsh, 2005; Kuramitsu *et al.*, 2007). These diverse microbial residents within the community display extensive interactions while forming organized biofilm structures, carrying out

sophisticated physiological functions, and inducing microbial pathogenesis under certain conditions (Kolenbrander *et al.*, 2002, 2010; Kuramitsu *et al.*, 2007).

The oral microflora has been implicated in many oral infectious diseases, including dental caries and periodontitis (Sigmund, 1979; Dahlen, 1993; Marsh, 1994; Tatsuji & Takeyoshi, 2004). The current concept proposes that in the case of complex microbiota, it is not merely the presence of a single organism in a complex community that determines the properties of a microflora, but it is the interactions between the resident bacteria that are crucial (Marsh, 1994, 2005). Such a microbial community-based pathogenic theory serves as a new concept for understanding the oral microbial community and the host in health and disease, as well as suggesting new strategies for disease treatment and prevention. With this new concept, it is crucial to study oral microbial flora as a whole, analysing its microbial composition as well as its physiological and biological properties.

The application of culture-independent, polymerase chain reaction (PCR) -based high-throughput methods, such as DGGE (Fujimoto *et al.*, 2003; Li *et al.*, 2005), terminal restriction fragment length polymorphism (Liu *et al.*, 1997), denaturing high-performance liquid chromatography (Barlaan *et al.*, 2005), and the metagenomic approach (Lazarevic *et al.*, 2009) have greatly facilitated community analysis by revealing its genetic diversity. However, conventional culture-dependent, *in vitro* methods are still indispensable in providing valuable information, including both phenotypic and genetic characterization of individual bacteria, as well as new physiological functions resulting from interactions among different microbial inhabitants within the community.

Many *in vitro* systems have been developed to study oral microbial community, such as constant-depth film fermentors (Kinniment *et al.*, 1996), saliva-conditioned flow cells (Cook *et al.*, 1998; Foster & Kolenbrander, 2004), and artificial mouths (Sissons *et al.*, 1991). Modified basal medium mucin (BMM), a peptone yeast extract-based medium supplemented with mucin (Sissons *et al.*, 1991, 1995), and defined medium mucin (Wong & Sissions, 2001), a chemically defined saliva analogue with mucin, are commonly used in these systems to support growth of the oral microbial community. Although the rates of growth in wet weight and the pattern in biofilm growth are similar to those of natural dental plaque (Sissons *et al.*, 1995), no detailed analysis has been performed to determine how the media may affect microbial community compositions through their selectivity, and the bias that might have been introduced because of the ill-represented microbial composition within cultured microflora. Currently, even with the advanced anaerobic culture techniques and available complex media for bacterial culture, <50% of oral bacterial species can be successfully culture (Kolenbrander, 2000; Wade, 2002), which has hampered our detailed understanding of the oral microbial world. A better medium with low selectivity would greatly facilitate *in vitro* study of the oral microbial community.

Traditionally, the development of new medium has largely relied on a trial-and-error methodology, which can be very time-consuming and costly. In this study, we used DGGE as an index to help us with medium development. DGGE is a PCR-based approach for microbial community analysis. It provides 'fingerprints' for each microbial flora and has been widely used to analyse environmental and human-associated microbial communities (Zijnge *et al.*, 2003; Nakatsu, 2007). Using the DGGE technique, we first analysed different profiles of oral microbial communities cultured in commonly used laboratory media. By comparing the profiles with that of the original oral sample, we selected a few media that were able to support the growth of certain salivary microbial subpopulations of which the combined profiles best represented the profiles of the original salivary microflora. The main active components from those selected media were then chosen as the ingredients for developing the new medium for culturing saliva-derived oral microbial flora.

This report describes the development of a new medium (SHI medium) for culturing saliva-derived oral microbial flora via the systematic applications of PCR-DGGE as an index. We demonstrated that, compared with other commonly used media, the newly developed SHI medium can sustain an *in vitro* microbial community with high diversity and similar microbial profiles to original saliva-derived oral microflora.

METHODS

Saliva collection

Saliva samples were collected from six healthy subjects, age 25–35 years. None was being treated for

any systemic disease or taking any prescription or non-prescription medication. Subjects were asked to refrain from any food or drink 2 h before donating saliva and to spit directly into the saliva collection tube, 5 ml saliva was collected from each person. Saliva samples were pooled together and centrifuged at 2600 *g* for 10 min to spin down large debris and eukaryotic cells. The supernatant was referred to as pooled saliva and used throughout this study. A 5-ml subsample of pooled saliva was centrifuged again at 14,000 *g* for 5 min and the pellet was collected for DNA extraction and PCR-DGGE analysis to obtain the bacterial profile of the original saliva. The rest of the pooled saliva was used for coating wells, seeding planktonic culture, and starting biofilms.

Culturing saliva-derived microbial flora using commonly used laboratory media

A 0.5-ml subsample of pooled saliva was inoculated into 5 ml of each of the following media: casitone–yeast extract broth (Difco, Franklin Lakes, NJ), Luria–Bertani broth (Difco), Todd–Hewitt (TH) broth (Difco), brain–heart infusion (BHI) broth (Difco), artificial saliva solution defined medium (He *et al*., 2008), BMM, tryptone–yeast extract gelatin–volatile fatty acids–serum (Ohta *et al*., 1986), sheep blood supplemented *N*-acetyl muramic acid (NAM) broth, Columbia broth (Teles *et al*., 2008) (Difco), cooked meat medium (Difco), peptone–yeast extract–glucose (PYG) medium, and chopped meat medium (Difco). The cultures were incubated under anaerobic condition (nitrogen 85%, carbon dioxide 5%, and hydrogen 10%) at 37°C for 24 h. Bacteria were collected by centrifugation at 14,000 *g* for 3 min and total genomic DNA was extracted using the MasterPure™ DNA purification kit (EPICENTRE, Madison, WI) for PCR-DGGE analysis.

SHI medium

The SHI medium has the following composition: proteose peptone (Difco) 10 g l^{-1}; trypticase peptone (Difco) 5.0 g l^{-1}; yeast extract (Difco) 5.0 g l^{-1}; KCl 2.5 g l^{-1}; sucrose 5 g l^{-1}; hemin 5 mg l^{-1}; vitamin K 1 mg l^{-1}; urea 0.06 g l^{-1}, arginine 0.174 g l^{-1}; mucin (type III, porcine, gastric; Sigma Chemical Co., St Louis, MO) 2.5 g l^{-1}; sheep blood (Colorado Serum Co., Denver, CO) 5% and NAM 10 mg l^{-1}.

Growth of saliva-derived biofilms

For crystal violet assay

A 2-ml subsample of pooled saliva was mixed with an equal volume of phosphate-buffered saline (PBS) and was centrifuged at 14,000 *g* for 3 min. Then, 200 μl supernatant was added to each well of the 24-well plate to pre-coat the wells and plates were incubated at 37°C with lid open for 1 h to dry the saliva coating. Plates were then sterilized under ultraviolet (UV) light for 1 h before 150 μl pooled saliva was inoculated into pre-coated well containing 850 μl BHI broth, TH broth or SHI medium. Plates were incubated at 37°C under anaerobic conditions to allow biofilm formation. After overnight growth, biofilms were evaluated by crystal violet assay. Three replicates were performed.

For confocal laser scanning microscopy microscopy

Two milliliters pooled saliva was mixed with an equal volume of PBS and was centrifuged at 14,000 *g* for 3 min. Then, 100 μl supernatant was added to each well of the eight-well chamber of the Lab-Tek® II Chamber Slide™ System (Nalge Nunc International, Naperville, IL). The objective slide was replaced by a thin cover-slide for proper confocal laser scanning microscopy (CLSM). Chambers were incubated at 37°C with lid open for 1 h to dry the saliva coating, and sterilized under UV light for 1 h. Then, 100 μl pooled saliva was seeded into each pre-coated eight-well chamber. Each chamber contained 300 μl TH broth or SHI medium. The chamber was incubated at 37°C under anaerobic conditions overnight before biofilm analysis by CLSM. Three replicates were performed.

Crystal violet assay

Quantification of biofilms was achieved by staining with crystal violet. Overnight biofilms were rinsed three times with PBS to remove the unattached bacteria. Then, 500 μl 0.5% crystal violet was added into each well and incubated at room temperature for 20 min before the solution was poured off and the wells were washed twice with PBS. After this, 300 μl 95% ethanol was added into each well and plates were incubated at room temperature with gentle shaking until no crystal violet was released from the biofilm. Ethanol solution was then transferred to a

new 24-well plate and biofilm mass was evaluated at an optical density of 595 nm using a micro-plate reader. Three replicates were performed.

The CLSM and image analysis

Overnight biofilms were rinsed three times with PBS to remove the unattached bacteria, biofilms were labelled using the LIVE/DEAD BacLight™ Bacterial Viability staining kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, biofilms were labeled with 1.67 μM SYTO9 (a green fluorescent dye that can cross intact membranes) and 10 μM propidium iodide (a red fluorescent dye that can only penetrate into cells that have lost membrane integrity). The biofilms were monitored through a 40× oil-immersion lens with a PASCAL LSM5 confocal laser scanning microscope (Zeiss, Jena, Germany). Green and red fluorescence was imaged sequentially to avoid cross-contamination of fluorescent signals. Image stacks of five randomly chosen spots were collected for each experimental sample, representative images are shown in the Results.

The CLSM images were analysed using the computer program COMSTAT (Heydorn *et al.*, 2000). Image stacks were converted to individual gray scale .tif images for each slice. Gray-scale images were converted into black and white, and compared with the original image to determine the threshold for the images, and the best value was chosen to give the most accurate conversion of the gray-scale to the black and white picture. The threshold value was fixed and then used for all image stacks. The image stacks of biofilm grown in TH broth and SHI medium were averaged and compared.

PCR-DGGE analysis

Total genomic DNA of bacterial samples were isolated using the MasterPure™ DNA purification kit (EPICENTRE). DNA quality and quantity were measured by a UV spectrophotometer at 260 nm and 280 nm. (Spectronic Genesys™, Spectronic Instrument, Inc. Rochester, NY).

Amplification of bacterial *16S* ribosomal RNA genes by PCR was carried out as described previously (Li *et al.*, 2005). Briefly, the universal primer set, Bac1 (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC TAC GTG CCA GCA GCC-3′) (Sheffield *et al.*, 1989) and Bac2 (5′-GGA CTA CCA GGG TAT CTA ATC C-3′) was used to amplify an approximately 300-base-pair (bp) internal fragment of the 16s ribosomal RNA gene. Each 50-μl PCR contains 100 ng purified genomic DNA, 40 pmol of each primer, 200 μM each dNTP, 4.0 mM $MgCl_2$, 5 μl 10× PCR buffer, and 2.5 U *Taq* DNA polymerase (Invitrogen). Cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, with a final extension period of 5 min at 72°C. The resulting PCR products were evaluated by electrophoresis in 1.0% agarose gels.

Polyacrylamide gels at an 8% concentration were prepared with a denaturing urea/formamide gradient between 40% [containing 2.8 M urea and 16% (volume/volume) formamide] and 70% [containing 4.9 M urea and 28% (volume/volume) formamide]. Approximately 300 ng of the PCR product were applied per well. The gels were submerged in 1× TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM ethylenediaminetetraacetic acid) and the PCR products were separated by electrophoresis for 17 h at 58°C using a fixed voltage of 60 V in the Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, the gels were rinsed and stained for 15 min in 1× TAE buffer containing 0.5 μg ml^{-1} ethidium bromide, followed by 10 min of de-staining in 1× TAE buffer. DGGE profile images were digitally recorded using the Molecular Imager Gel Documentation system (Bio-Rad Laboratories).

454 Pyrosequencing

Bar-coded (approximately 10–12 bp long) 16S primers 27F (AGAGTTTGATYMTGGCTCAG) (Edwards *et al.*, 1989) and 534R (ATTACCGCGGCTGCTGG) (Muyzer *et al.*, 1993) were used for PCR amplification of the 16S ribosomal RNA V3 region from the extracted DNA. The degenerate bases Y (nucleotide C or T) and M (nucleotide A or C) were incorporated into the primers and the PCR products were cleaned up using Qiaquick column purification kit (Qiagen Inc., Valencia, CA). The amplicons were normalized, pooled, and library construction was completed using titanium chemistry (Roche Diagnostics, Inc., Basel, Switzerland). The average length of sequencing products was about 253 bp, solid-phase reversible immobilization clean up was performed using Ampure XP

beads (Beckman Coulter Inc., Brea, CA) to ensure that the target amplicons with size of 507 bp were used for library preparation. The adaptor primers A (5′-CCATCTCATCCCTGCGTCTCTCCGAC-3′) and B (5′-CCTATCCCCTGTGTGCCTTGGCAGTC-3′) were ligated to both the 5′ and 3′ ends of the amplicons, before they were attached to an emulsion PCR bead for amplification before 454 pyrosequencing. Using each sample's individual barcodes, the 454 sequence data were de-convolved into the respective samples. After trimming the bar codes and removing low-quality sequences, taxonomic assignments of the sequences were made using the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007; Cole *et al.*, 2009), which classifies sequences to the genus level. After filtering, a total of 11,692 reads for the original saliva and 9971 from the *in vitro* grown sample were used in the analyses. Those 454 sequences classified as belonging to the *Streptococcus* genus were analysed further to identify their nearest neighbors in the RDP database. This was done by searching these sequences against all *Streptococcus* sequences in the RDP database using BLAST.

Statistical analysis

Significance of differences between average values was analysed by Student's *t*-tests using MS Excel.

RESULTS

Survey of the commonly used laboratory media for their ability in culturing oral microbiota

One of the limiting factors in *in vitro* studies of the oral microbial community is the selectivity of culture media, which often result in the microbial diversity of oral community being poorly represented. In an effort to systematically survey the ability of different laboratory culture media to support the growth of the oral microbiota, 12 current commonly used laboratory media were chosen to culture human pooled salivary samples, including BMM and NAM, two of the frequently used media to sustain oral microbes. The microbial diversity within each medium was analysed using PCR-DGGE as the profiling tool. Results showed that, compared with original saliva, bacterial samples from all the tested media displayed various levels of reduction in the number of bands, suggesting a decreased microbial diversity when using these media to culture salivary microbes. There were striking differences in the banding patterns between samples from different media, indicating that each medium had selectivity for certain bacterial sub-populations within the community (Fig. 1).

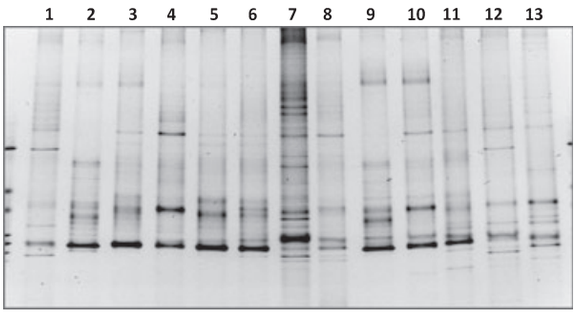

Figure 1 Polymerase chain reaction denaturing gradient gel electrophoresis analysis showing the bacterial profiles of saliva-derived overnight planktonic culture in different media. Lane 1, peptone–yeast extract–glucose; lane 2, artificial saliva solution; lane 3, Columbia; lane 4, tryptone–yeast extract–gelatin–volatile fatty acids–serum; lane 5, Todd–Hewitt; lane 6, brain–heart infusion; lane 7, original saliva; lane 8, Luria–Bertani; lane 9, casitone–yeast extract; lane 10, chopped meat medium; lane 11, cooked meat medium; lane 12, basal medium mucin; lane 13, sheep-blood-supplemented *N*-acetyl muramic acid broth. Three replicates were performed and a representative gel image is shown.

SHI medium displayed the least selectivity in culturing oral microbiota from pooled human saliva

The PCR-DGGE analysis indicated that the media tested had various levels of selectivity in supporting the growth of saliva-derived oral microbiota. Figure 1 also revealed that although the individual banding patterns from PYG, BMM and NAM were different, if combined, the cumulative banding pattern was very close to that of the original saliva. We reasoned that combining the main nutritional ingredients from those three media could result in a new culture medium that might be able to support more diversified bacterial growth. Using this as a guideline, we developed a new medium, named SHI medium, for culturing the oral microbiota.

Using proteose peptone, trypticase peptone and yeast extract as the basic components, we developed

SHI medium by supplementing the basic components with mucin, hemin, vitamin K, urea, and arginine, which are the active ingredients of BMM medium. Two main supplements of the NAM broth, sheep blood and *N*-acetylmuramic acid were also included in the new medium.

PCR-DGGE analysis revealed that SHI medium was able to sustain a microbial community with a profile very similar to that of the original saliva, suggesting that compared with other commonly used laboratory media, SHI medium had the least selectivity in culturing saliva-derived oral microbiota (Fig. 2).

454 pyrosequencing analysis

To further confirm that the newly developed SHI medium was indeed able to support the growth of diversified bacterial species within salivary samples, we performed 454 pyrosequencing analysis on SHI medium-grown samples and the original salivary samples. Results showed that bacterial species of at least 40 different genera, which fall into eight bacterial divisions, were detected from the original saliva samples, with Firmicutes, Proteobacteria and Bacteroidetes being the most dominant phyla. Most excitingly, the microbial profile of the salivary flora grown on SHI medium displayed a striking similarity to that of the original sample at the genus as well as at the species level, including microbes within the phylum TM7 (Figs 3 and 4).

Comparison of dominant sequences falling within the *Streptococcus* genus for the original saliva flora and the SHI medium-cultivable saliva flora showed that all the *Streptococcus* spp. within the saliva sample, both cultured and previously listed as uncultured *Streptococcus* spp., could be recovered by culturing on the newly developed SHI medium (Fig. 4).

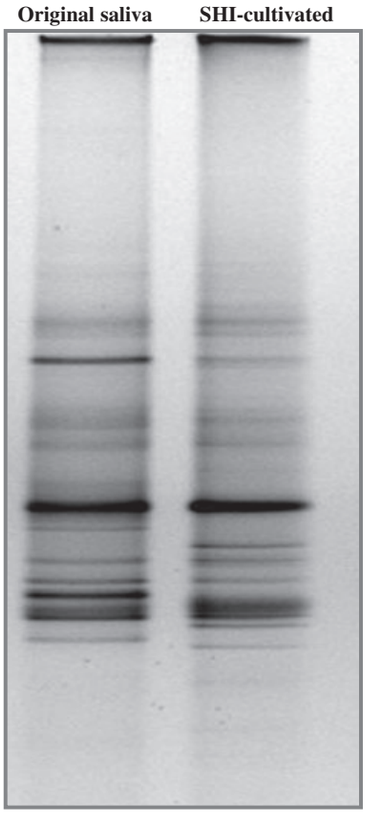

Figure 2 The bacterial profile of original pooled saliva and the microbial profile of saliva-derived overnight planktonic culture in newly developed SHI medium. Three replicates were performed and a representative gel image is shown.

SHI medium allowed better biofilm formation of saliva-derived microbes than TH and BHI media

The SHI medium was able to support more diversified microbial growth than other commonly used laboratory media. We also wanted to assess the biofilm formation of salivary microbes when grown on different media. Crystal violet staining showed that biofilm in SHI medium gave the highest optical density reading at 595 nm, which was significantly higher than those grown in BHI or TH media ($P < 0.05$); whereas there was no difference between biofilm samples in BHI and TH media ($P > 0.1$) (Fig. 5). The result indicated that compared with BHI and TH media, SHI medium was allowed better biofilm formation of saliva-derived microbes.

CLSM imaging and COMSTAT analysis of saliva-derived biofilms grown on different media

Results presented above implied that, compared with other commonly used laboratory media, SHI medium was superior for growing saliva-derived oral microbiota in terms of the microbial diversity and biofilm it was able to support. To confirm this observation, we performed the CLSM imaging on the saliva-derived biofilm grown in different media. CLSM imaging revealed that saliva-derived biofilm grown in TH medium was sparse with less surface coverage

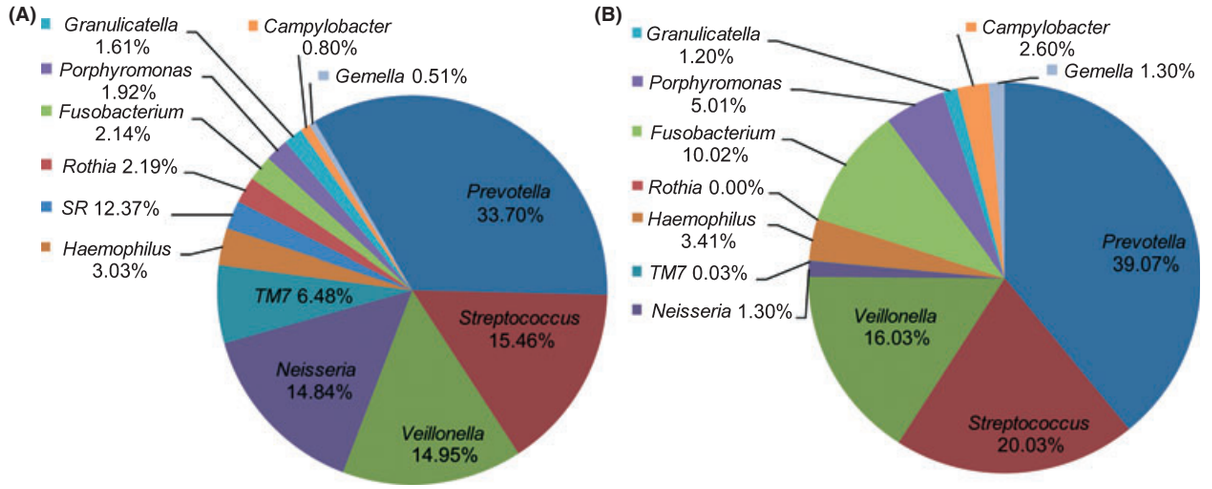

Figure 3 Comparison of original salivary and SHI medium cultured salivary bacterial species distribution using 454 pyrosequencing. Taxonomic assignments of the sequences were made using the Ribosomal Database Project which classifies sequences to the genus level. The community profile of the dominant members from (A) the original pooled saliva and (B) the *in vitro* grown community in SHI medium.

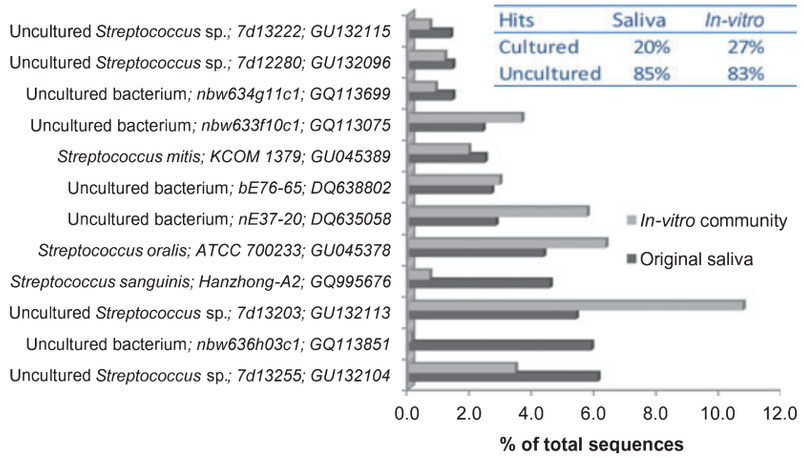

Figure 4 Comparison of dominant sequences falling within the *Streptococcus* genus for the original saliva and the *in vitro* SHI medium-cultured community. For this analysis, all *Streptococcus* 16S ribosomal RNA gene sequences of cultured and uncultured strains were downloaded from the Ribosomal Database Project (RDP). Each 454 sequence that was classified as belonging to the *Streptococcus* genus, was assigned to the best matching *Streptococcus* RDP sequence. A total of 295 and 296 unique sequences for *Streptococcus* were observed for the original saliva and *in vitro* grown sample, respectively. Total S*treptococcus* sequences matching the cultured and uncultured strains for each sample are shown in the inset.

and much thinner compared with biofilms grown in SHI medium (Fig. 6A,B). COMSTAT analysis showed that the biofilm grown in TH medium had an average biovolume of $5.8 \pm 1.67\ \mu m^3\ \mu m^{-2}$; while the average biovolume of SHI medium-cultured biofilm was $19.71 \pm 3.42\ \mu m^3\ \mu m^{-2}$ (Fig. 6C). Statistical analysis indicated that the difference in biovolume between the two media was significant ($P < 0.001$). Furthermore, after overnight growth in TH medium, more than 10% of the bacterial cells within biofilm were dead (red-stained), whereas dead cells made up <5% of the total bacterial population within SHI medium-cultured biofilm. The result is consistent with crystal violet staining data, suggesting that SHI medium was able to sustain the growth of saliva-derived biofilm with higher biomass.

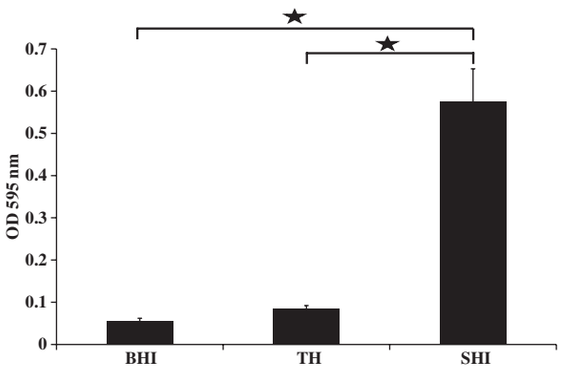

Figure 5 The crystal violet assay to assess overnight biofilm formation of saliva-derived microbes cultivated in brain–heart infusion, Todd–Hewitt or SHI medium. Three replicates were performed for each assay. Average values ± SD are shown; the star indicates significant difference ($P < 0.05$) between the two values.

DISCUSSION

Developing media for growing oral microbial flora has always been a challenge. This is largely because of the range of bacterial species residing within the oral cavity and their different nutritional requirements (Kolenbrander, 2000).

Saliva has been used in many *in vitro* systems as a nutrient source for sustaining the oral microbial flora (De Jong & Van Der Hoeven, 1987; Palmer *et al.*, 2001; Foster & Kolenbrander, 2004). However, the use of natural saliva has many disadvantages: it is difficult to collect and sterilize in large quantities, and is variable in composition and properties. Furthermore, because of its limited carbohydrate content, saliva does not support the growth of complex oral microbiota. Several media have been developed to try to enhance the growth of complex oral microbial communities. In an effort to increase the growth yield of *in vitro* culture of a dental plaque-derived oral microbial community, mucin, the principle glycoprotein in saliva, was added to the BM broth, a medium that had originally been developed for growing *Bacteroides* spp. isolated from the human oral cavity (Shah *et al.*, 1976). The mucin-containing new medium (BMM) greatly increased the growth yield, largely as a result of the increased number of anaerobes (Gle-

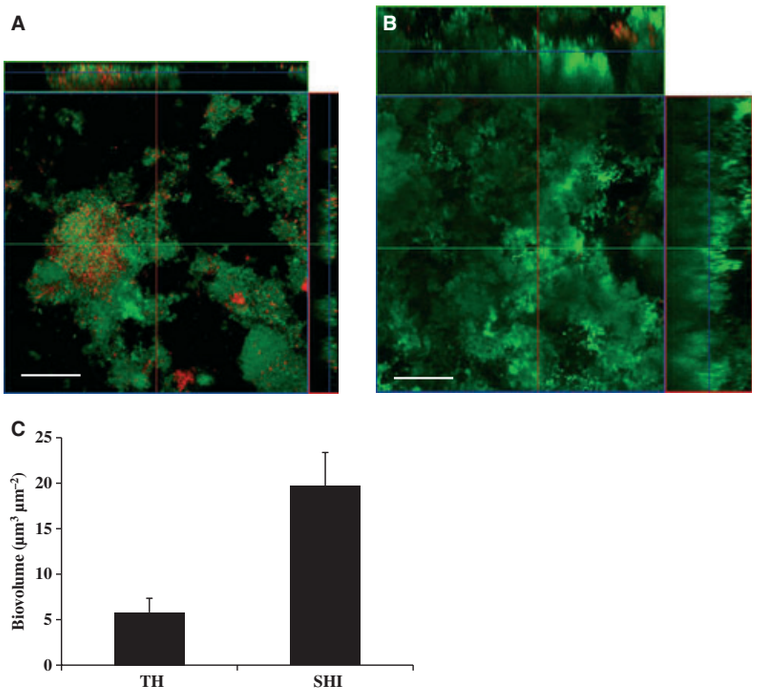

Figure 6 Comparison of saliva-derived biofilms grown in Todd–Hewitt (TH) or SHI medium visualized by confocal laser scanning microscopy. Z-stack image of saliva-derived biofilm (live/dead stained) grown in (A) TH medium and (B) SHI medium. Biovolume of biofilms formed using different media was calculated (C) using the software COMSTAT. Images of five randomly chosen spots were collected for each experimental sample, representative images are shown. Error bar in the calculated biovolume is standard deviation. Scale bar = 50 μm.

nister *et al.*, 1988). However, the recovery of certain oral species, such as *Veillonella* spp. decreased. Later, Wong *et al.* developed a chemically defined analogue of saliva (defined medium mucin) in an effort to substitute for BMM complex medium (Wong & Sissions, 2001). They demonstrated that defined medium mucin-grown dental plaque can achieve realistic growth rates and patterns similar to that obtained by BMM. Another frequently used medium for the culture of oral microbes is NAM (*N*-acetyl muramic acid) broth. It contains NAM, a monosaccharide derivative of *N*-acetylglucosamine, which has been shown to facilitate the growth of certain subgingival anaerobic bacteria, such as *Tannerella forsythia* and *Porphyromonas gingivalis* (Teles *et al.*, 2008).

However, there has been no detailed analysis to compare the microbial profiles of medium-cultivated and original dental plaque or saliva samples so it is not clear how selective these media are in supporting the oral community and whether the cultivated microbial flora can best represent the genetic diversity within plaque or saliva samples. In an effort to survey the ability of different media to sustain the growth of oral microbes, we chose commonly used laboratory media, including BMM and NAM, to cultivate salivary microbial flora. PCR-DGGE analysis revealed that none of the media tested was able to support a community that maintains high diversity and a similar community profile to the original microbial flora within saliva samples (Fig. 1).

The traditional trial-and-error method for developing new medium also made the process very time-consuming and less efficient. In this study, we used DGGE as an index to help us to develop new medium for best sustaining the genetic diversity of the oral community. By comparing the DGGE profiles of microbial flora cultured in the tested media with that of the original samples, we found that although the microbial patterns of individual culture on PYG, BMM and sheep blood-supplemented NAM media were different, if combined, the cumulative pattern was very close to that of the original saliva sample. Based on this observation, we proposed that by adding the critical ingredients of the three media, we might be able to develop a new medium that better met the nutritional requirements of a wider range of salivary microbes and so yield a cultivable flora that would better represent the microbial diversity within the salivary sample.

Using this as a guideline, we developed SHI medium by adding critical supplements from these broths to the base ingredients, which include peptone and yeast extract, the main components of the chosen media. Mucin and hemin from BMM were included in the new medium. Mucin is the principle glycoprotein of saliva and has been shown to be an important growth-limiting substrate for the complex oral microflora (Glenister *et al.*, 1988). Hemin has been shown to stimulate the growth of a variety of oral species, including cocci, rods and filamentous bacteria (Gilmour & Poole, 1970). As a nutrient-rich supplement, sheep blood was also added to the new medium to facilitate the growth of fastidious and slow-growing, obligate anaerobic bacteria within the oral flora. Another main supplement is *N*-acetyl muramic acid from NAM broth, which has been shown to enhance the growth of certain gram-negative, anaerobic subgingival bacteria such as *Tannerella forsythia* (Wyss, 1989). Lastly, sucrose was used as a substitute for glucose, an ingredient from PYG, and it was shown to achieve a better recovery of the streptococci from the salivary samples than glucose (data not shown).

The DGGE analysis showed that the new SHI medium sustained the growth of a microbial community with a profile closer to that of the original samples (Fig. 2). The result of 454 pyrosequencing strongly indicate that SHI medium is able to maintain an *in vitro* salivary microbial community with high diversity and similar microbial profiles at both genus and species levels (Fig. 3). Importantly, the *in vitro* community, like the saliva sample, has a high percentage of uncultured representatives (85 and 83%, respectively) (Fig. 4). Crystal violet staining and CLSM analysis of the biofilm showed that compared with commonly used laboratory media such as TH broth, SHI medium was superior in yielding more biomass when used to culture saliva-derived biofilm (Figs 5 and 6).

The *in vitro* community also contains several of the candidate phylum TM7 (0.06% of total), which have been referred to as biology's 'dark matter' problem (Marcy *et al.*, 2007). This phylum is a major focus of study because although they have been identified (via clone sequences) in a wide variety of habitats, researchers have yet to obtain a stable culture of any isolate. A number of novel approaches have been used to investigate TM7 organisms, including the use of microfluidic devices (Marcy *et al.*, 2007) and cell separation by fluorescent *in situ* hybridization and

flow cytometry (Podar *et al.*, 2007) to obtain single cells for whole genome sequencing. The data presented here show that representatives of TM7 are actively growing in the *in vitro* environment and this may allow the first study of their biological function in a stable mixed community.

As with all other media, the newly developed SHI medium also displayed a certain level of selectivity. For example, based on 454 pyrosequencing analysis, sequences with hits in SR1 division, which accounted for 2.3% of the total sequence hits in original saliva samples, were lost when grown in SHI medium. At the same time, some minor species within the original saliva samples, such as *Treponema*, *Caenibacterium*, and *Oribacterium*, which accounted for <0.2% of the total sequence hits, also could not be recovered from *in vitro* cultivation (data not shown). Nevertheless, the high degree of overlap between the two profiles at genus as well as species levels indicated that SHI medium could be useful in establishing a representative model oral community, and facilitating the study of previously un-culturable oral bacterial species, such as TM7. Finally, it is reasonable to suggest that this DGGE-guided method could be used to develop novel media not only for oral microbial communities but also for other complex microbial communities.

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