Microbial community interactions: effects of probiotics on oral microcosms
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Chapter III - MLPA diagnostics of complex microbial communities: relative quantification of bacterial species in oral biofilms

This chapter has been published as:

ABSTRACT
A multitude of molecular methods are currently used for identification and characterization of oral biofilms or for community profiling. However, multiplex PCR techniques that are able to routinely identify several species in a single assay are not available. Multiplex ligation-dependent probe amplification (MLPA) identifies up to 45 unique fragments in a single tube PCR. Here we report a novel use of MLPA in the relative quantification of targeted microorganisms in a community of oral microbiota. We designed nine species specific probes for Actinomyces gerencseriae, Actinomyces naeslundii, Actinomyces odontolyticus, Candida albicans, Lactobacillus acidophilus, Rothia dentocariosa, Streptococcus mutans, Streptococcus sanguinis and Veillonella parvula; and genus specific probes for selected oral streptococci and lactobacilli based on their 16S rDNA sequences. MLPA analysis of DNA pooled from the strains showed the expected specific MLPA products. Relative quantification of a serial dilution of equimolar DNA showed that as little as 10 pg templates can be detected with clearly discernible signals. Moreover, a 2 to 7% divergence in relative signal ratio of amplified probes observed from normalized peak area values suggests MLPA can be a cheaper alternative to using qPCR for quantification. We observed 2 to 6 fold fluctuations in signal intensities of MLPA products in DNAs isolated from multispecies biofilms grown in various media for various culture times. Furthermore, MLPA analyses of DNA isolated from saliva obtained from different donors gave a varying number and intensity of signals. This clearly shows the usefulness of MLPA in a quantitative description of microbial shifts.

INTRODUCTION
The human oral cavity harbors a multifarious group of microorganisms that forms a complex community and occupies diverse specific niches and microenvironments. The resident oral microflora is sustained in an apparent state of balance or microbial homeostasis, once microorganisms are established in the mouth (MARSH 2003a). Environmental perturbations change the dynamic ecology of the resident, evoking microbial shifts in terms of concentrations and activities, which at times may result
in the development of cariogenic and periodontal diseases (MARSH 2000; MARSH 1994). Dental plaque, a surface bound community which is regarded as a biofilm is of particular importance in the ecology of the oral microbiota. The formation of plaque or growth of biofilm on dental surfaces by rapid colonization of bacteria follows a particular microbial succession, which is largely dependent on the host’s genotype, quality of the immune system, diet, general hygiene and health conditions (MARSH 2003a; PALMER et al. 2007). Growing in a biofilm gives certain advantages to the resident microflora (SCHIE IE and PETERSEN 2004). The biofilm life style enables bacteria to develop mechanisms that minimize the effect of antimicrobials and the human immune defense system (GILBERT et al. 1997; MAH and O’TOOLE 2001).

About half of the more than 700 bacteria species inhabiting the oral cavity are culturable, though this assumption is made by studying mainly sub-gingival samples (AAS et al. 2005; PRAT TEN et al. 2003). Traditional culturing methods and biochemical assays thus do not allow to fully characterizing the inhabitants of the oral cavity. Instead, molecular techniques provide the ideal tools for identification and characterization of bacteria that are hitherto undiscovered and most of the traditionally uncultivable bacteria. Current determination of the microbial etiology of dental diseases which utilizes both the culturing and molecular methods is far from being adequate (MARSH 2003b). Similarly, quantification of the bacteria in biofilms is a daunting task. The change in the dynamics of the resident flora due to changes in the environment poses a challenge for accurate determination of the spatial and temporal abundance of the species. In vitro models show ways of evaluating the shift in populations associated with the onset of common oral diseases (DALWAI et al. 2006). Real-time PCR based methods such as the Taq-man system, qPCR and checkerboard DNA-DNA hybridization are shown to quantify oral bacteria in biofilms (DALWAI 2007; MARTIN et al. 2002; NADKARNI et al. 2002; SOCRANSKY et al. 2004; SUZUKI et al. 2004a; SUZUKI et al. 2005; SUZUKI et al. 2004b). Arrays of molecular methods are used for identification and characterization of oral biofilms or for community profiling. Various multiplex PCR techniques targeting the 16S rDNA gene (TRFLP, DGGE, LH-PCR), southern blotting, genomic fingerprinting using random PCR are so far the most widely used. However, these
commonly used methods all have their shortcomings. For instance, southern blotting is cumbersome and time-consuming; PCR generates false positives; restriction site based techniques are ponderous to perform; genomic fingerprinting generates arbitrary data and a metagenomics approach is time consuming and requires expensive equipments. Thus, there is a need to develop a simpler, faster and cost effective method for a relative quantification of a selected set of bacteria in multispecies consortia at different time points and at different nutritional and environmental conditions.

The multiplex ligation-dependent probe amplification (MLPA) method (Schouten et al. 2002) is widely used to identify genes in various human genetic disorders that result in different diseases. This method is proven to identify these genetic alterations whether they are a result of point mutations, insertions, deletions, duplications or recombination events (Hogervorst et al. 2003; Stern et al. 2004; Volikos et al. 2006; Vorstman et al. 2006; Wiltgen et al. 2006). Notwithstanding the complex process of designing probes, the elegance and simplicity of this method makes it applicable to any type of DNA. Recently an MLPA based assay which is able to identify 15 respiratory viruses has been described (Reijans et al. 2008). The only MLPA based assay on bacteria we are aware of is the recent characterization of Mycobacterium tuberculosis using drug resistance markers for identification (Bergval et al. 2008). A ligase chain reaction (LCR) (Wiedmann et al. 1994) based method, which is similar to MLPA, was shown to detect multiple blood-borne bacterial pathogens in a single assay (Pingle et al. 2007). The aim of this study was to investigate the use of MLPA to screen the composition and dynamics of complex microbial communities, using oral biofilms as an example, and to develop an easy to perform and cost effective multiplex assay for the relative quantification of a selected set of bacteria in a single reaction. Our report here shows the application of MLPA in simultaneously identifying targeted oral bacteria in a specific manner from pure cultures, consortia of known species, as well as DNA obtained from biofilm and saliva samples. We also show the use of MLPA in detecting the shifts in bacterial composition in biofilms when the ecology of the oral microbiota is changed due to changes in the environment.
MATERIALS AND METHODS

Sample collection and preparation

A panel of eight oral bacterial species was chosen for the MLPA assay (Table 1). These species were selected because they are frequently found in dental biofilms and the presence of most of these is associated with dental caries (Fiocco et al. 2007b). Biofilms were grown in a constant depth film fermentor (CDFF) using saliva from a single source as inoculum as described (Deng et al. 2005; Wilson 1999) or in a consortium in 20 mL Bijou tubes. Stimulated saliva was collected from six healthy subjects. The saliva collected was filtered through a sterilized glass-wool and diluted in 20 mL glycerol in a sterile tube, and then stored at -80 °C. To inoculate the CDFF, thawed saliva was mixed together with 200 mL of artificial saliva medium (mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L; KCl, 0.2 g/L; CaCl₂, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K₁, 0.0002 g/L) (McBain et al. 2005). The mixture of saliva and artificial medium was pumped into the CDFF at the flow rate of 2.4 mL per minute in conventional mode of the CDFF as originally designed (Wilson 1999). The inoculation was settled for 1 h, and then the medium was supplied at flow rate 0.3 mL per minute for 3 hours by using the back-and forth mode of the CDFF as described (Deng et al. 2005). The sucrose pulse was supplied 8 times per day at flow rate 1.2 mL per minute for 5 minutes and then continued with pumping the medium. The biofilms grown in the CDFF were collected after 14 days.

In a parallel study, a biofilm was formed in 20 mL Bijou tubes (Grenier Bio-one, Frickenhausen, Germany) from a consortium of eight species (A. gerencseriae, A. naeslundii, A. odontolyticus, C. albicans, L. acidophilus, S. mutans, S. sanguinis and V. parvula). The strains were grown on blood agar first and then half a loop of the pure culture was transferred to 2 mL artificial saliva medium (McBain et al. 2005) and grown at 37 °C anaerobically overnight. 1.5 mL of the fully grown culture from each species was then added to sterile 20 mL Bijou tubes and mixed vigorously. Then, 0.6 mL of the consortium mix was grown in 20 mL Bijou tubes containing three different media (10 mL) - artificial saliva alone, artificial saliva supplemented with 0.2% sucrose and artificial saliva supplemented with 0.2% sucrose and PIPES.
(50 mM). The medium was refreshed after 24 h and 48 h incubation. Biofilm was collected 7 h, 24 h and 48 h after incubation at 37 °C.

**DNA isolation**

DNA from individual strains was extracted from fully grown cultures with a concentration of 10⁶ - 10⁷ colony forming units (cfu) per mL using the FastDNA® SPIN® Kit (For Soil) (Q-BIOgene) according to the manufacturers’ instruction. Community DNA from multispecies saliva or CDFF grown biofilms samples was obtained after bead beating followed by using DNeasy Blood & Tissue Kit (Qiagen). More specifically, biofilms were resuspended in 1 mL ATL buffer (Qiagen) and transferred to a bead beating vial containing 0.5 gram of 0.1 mm sterilized glass beads (Biospec products, Inc.). Bead beating was done at 5.5 m/s for 30 sec. (Fast prep Qbiogene). The mix was then centrifuged at 14,000 x g for 5 minutes. From this step onwards we followed the DNeasy Blood & Tissue Kit instruction manual. We also isolated DNA from the eight species biofilm/consortium (A. gerencseriae, A. naeslundii, A. odontolyticus, C. albicans, L. acidophilus, S. mutans, S. sanguinis and V. parvula) sample grown in a single tube the same way as from saliva and CDFF biofilms. All DNAs were treated with RNAse to avoid RNA interference with probe hybridization.

**MLPA probe design**

The MLPA probes we designed target the various regions of the 16S rDNA molecule (Table 1), except for the S. sanguinis probe, which is based on 23S rDNA. Full MLPA probe amplification is only possible after successful hybridization of the two oligonucleotides half-probes adjacent to each other on the target DNA and subsequent ligation to one another. The ligation of the two probes is very specific and can only occur when the probes are exactly bound to their specific complementary target. Any sequence dissimilarities, e.g., single nucleotide polymorphisms (SNPs) at the ligation site, will prevent proper ligation and will result in the absence of a peak signal.
Initially we designed synthetic probes following the guidelines of MRC-Holland (Amsterdam, the Netherlands). Briefly, the criteria for designing probes require a minimum length difference between probes - 4 nucleotides. Tm of each hybridizing sequence, Left Probe Oligonucleotide (LPO) or Right Probe Oligonucleotide (RPO): $\geq 70^\circ\text{C}$. $\Delta G$ (secondary structure): $\geq 0$. LPO with preferably a maximum of 2 G/C directly adjacent to the ligation site and preferably a maximum of 3 G/C directly adjacent to the primer were chosen. No more than 7 nucleotide overlap was allowed between probes to avoid competition. Genus and species specific probes were designed manually using a global alignment of 930 sequences from oral samples obtained from the ribosomal database project RDP database. The probe match program from RDP (http://rdp.cme.msu.edu/probematch/search.jsp) was used to determine the specificity of the probes. In addition, we used the BLAST algorithm to inspect the uniqueness of our selected probe sequences. Both the LPOs and RPOs were synthesized by Biolegio (Nijmegen, the Netherlands). Once we determined that specific signals are obtained from our probes, the RPOs were manufactured by MRC-Holland using the M13-cloning based production strategy as described previously (SCHOUTEN et al. 2002).
Multiplex ligation-dependent probe amplification

Table 1. Strains and probes used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Probe*</th>
<th>MLPA product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces naeslundii (ATCC 12104)</td>
<td>CGAACGGTGAAAGGGGCTCTTGTTGTTGGGCTCTGGAAT</td>
<td>356</td>
</tr>
<tr>
<td>Actinomyces gerencseriae (ATCC 23860)</td>
<td>GAGTGCCAGACGGTGGTGCTTACGTA (74 - 159)</td>
<td>202</td>
</tr>
<tr>
<td>Actinomyces odontolyticus (ATCC 17929)</td>
<td>CCCGGTGTGAAACCTTTTCTTTTCTCTGCTACGTAAGCCGCA</td>
<td>364</td>
</tr>
<tr>
<td>Candida albicans (CBS 8575)</td>
<td>ACTCAAGGGTTGGTGAGG (485 - 546)</td>
<td>191</td>
</tr>
<tr>
<td>Lactobacillus acidophilus (ATCC 4356)</td>
<td>GAGATTCGCTTGCTCCAGCCTTCGCTTACGTA</td>
<td>314</td>
</tr>
<tr>
<td>Rothia dentocariosa (ATCC 17931)</td>
<td>CGGACGACGGCTTTCCCATGGCTTACGTA (1098 - 1131)</td>
<td>171</td>
</tr>
<tr>
<td>Streptococcus mutans (ATCC 25175)</td>
<td>GCTATGGCCTACACATGTGTGCTCTGCTACTTACGTA</td>
<td>301</td>
</tr>
<tr>
<td>Veillonella parvula (ATCC 10790)</td>
<td>CCGTGAATCTGCTTGCTTACGTA (675 - 753)</td>
<td>252</td>
</tr>
<tr>
<td>Streptococcus sanguinis (ATCC 10556)</td>
<td>CCGTGAATCTGCTTGCTTACGTA</td>
<td>126</td>
</tr>
<tr>
<td>streptococci common**</td>
<td>ACTTACGCTGCAAGGGGAGATGGAATCCCATGTA (1098 - 1131)</td>
<td>152</td>
</tr>
<tr>
<td>lactobacilli common***</td>
<td>CCTTACGCTGCAAGGGGAGATGGAATCCCATGTA</td>
<td>326</td>
</tr>
<tr>
<td>Universal 1</td>
<td>GTGAAATCGCTCCAGGCGGCTTACGTAACAGCCGGCTTACGTA (1458 - 1521)</td>
<td>237</td>
</tr>
<tr>
<td>Universal 2</td>
<td>CGGAGGAAGGCTCCCATGTAACAGCCGGCTTACGTA</td>
<td>185</td>
</tr>
</tbody>
</table>

L. fermentum (ATCC14931)  
L. plantarum (SA-1)  
L. salivarius (ATCC 11741)  
S. anginosus (ATCC 33397)  
S. mitis 2 (SK 149)  
S. parasanguinis (M1021)  
S. sobrinus (OIH)  

* - Only hybridizing sequences included. Left probe oligonucleotides (plain), right probe oligonucleotides (bold). Numbers in brackets indicate nucleotide positions in E. coli.


MLPA reactions on bacterial DNA were performed with a slight modification of the standard protocol for human DNA. Routinely 50–500 pg of DNA from pure cultures or 500 pg–10 ng from saliva and biofilm samples was used. All the MLPA reagents were provided by MRC-Holland (Amsterdam, the Netherlands). The whole MLPA protocol was performed in T1-gradient thermo cycler (Biometra GmbH, Göttingen, Germany) according to the standard MLPA protocol http://www.mlpa.com/pages/support_mlpa_protocolspag.html. The PCR products were separated by capillary electrophoresis using either the CEQ 800 genetic analyzer (Beckman instruments) (Cy5 labeled primers were used) or the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) (primers were labeled with FAM).

Probe specificity and validation of signal quantification

We performed experiments where either DNA from a single species or pooled DNA of equimolar proportions from all or part of the strains under study is hybridized against a varying combination of probe mixes containing either single pairs of (LPO and RPO) or mixed pairs with varying combinations.

Template DNAs (100 pg) were spiked with varying amounts of foreign DNA obtained from human and other related bacteria against which only the universal probes but not the species specific ones are hybridized. This was to test the minimum amount of DNA detectable when standard conditions are used and to assess risk of contamination. In addition, to check the possible cross-reactivity of probes, species specific probes were tested on DNA obtained from closely related species with a high degree of homology with the 16S rDNA sequences. The following cross-reactivity tests were done. S. mutans probe was tested against Streptococcus parasanguinis, Streptococcus sanguinis, Streptococcus mitis 2, Streptococcus anginosus and Streptococcus sobrinus; the L. acidophilus probe was tested against Lactobacillus salivarius, Lactobacillus fermentum and Lactobacillus plantarum; the Actinomyces species probes were tested against each other.
Serial dilution of pooled DNA (5 ng – 30 pg), from strains whose probes are based on 16S rDNA, were used as template to test the validation of quantifiable signals obtained from the reactions. A control plasmid (33 zmol) that contains the target sequences of two probes producing 141 and 237 bp length fragments was added to the reactions as an internal control. These control probes, unlike the DQ controls are subject to all parameters affecting the result of the MLPA reaction. They are used to test efficiency of hybridization, ligation and PCR amplification of ligated probes. They are also used as internal standards to normalize signal intensities for relative quantification. The resulting signals (peak area) were normalized as described previously (SCHOUTEN et al. 2002). The reactions were done in triplicate; the intensity of signals was normalized by dividing signal intensity of each probe to the sum of all signals from the control probes in each dilution series. This relative signal was in turn divided by the signal ratio of its own control probe deduced from the average value of triplicate runs. The statistical significance of the data was investigated by calculating the standard deviation for the triplicate in each data series as well as performing a general linear model repeated measures test (GLM-RM) in SPSS (version 14).

**Community profiling with DGGE**

We employed DGGE using the primers described by Muyzer et al (MUYZER et al. 1993) to investigate the microbial shifts occurring when biofilms were produced under different environmental conditions. We tested a biofilm (consortium) containing eight species grown in a single tube where samples were grown for 7 h, 24 h and 48 h in three different media. DGGE was performed using the Bio-Rad D-Code system (Bio-Rad, Herculas, CA) as described by Muyzer et al (MUYZER et al. 1993). The gel image was processed and quantification of bands was performed using the GelCompar II version 4.0 software package (Applied Maths, Kortrijk, Belgium). The relative band intensities were calculated using the markers as a reference and then compared with those obtained from the MLPA experiments.
RESULTS

MLPA

The initial tests of MLPA using synthetic probes showed that specific signals from each sample can be obtained. However, the signals contained shoulder peaks which made size calling tedious and problematic. After confirming that MLPA works using synthetic probes, we finally reverted to using the M13-derived probes. This strategy enabled us to add stuffer sequences with varying lengths at the right probe oligonucleotide (RPO). This in turn allows the production of up to 45 probes which contain sufficient size differences to be separated by other means, for instance gel electrophoresis, rather than by capillary electrophoresis. In addition we found that the peaks generated by these probes were sharper and contained almost no shoulder peaks (Figure 1).

We were able to generate specific amplified probe fragments using the probes that were designed for these strains. A mixed sample containing equimolar DNA pooled from the eight strains in the study produced all the specific MLPA products, as well as the common probes directed to a group of streptococi and lactobacilli, and the control “universal” probes designed to detect most of the oral bacteria (Figure 1). Only a single specific MLPA product is obtained when a template DNA from a single species is hybridized with a probe mix that contained all of the probes (Figure 2). The cross reactivity tests on the S. mutans, L. acidophilus and the Actinomyces spp. did not produce any MLPA product other than those that are generated from universal or from group specific probes. We also observed that as little as 10 pg of template DNA can be detected with discernible signal, depending on the fidelity or reproducibility of probes, which is dependent on their length, folding capacity, Tm, sequence uniqueness and the oligo quality.

The effect of variations in copy number and probe fidelity was investigated by performing MLPA on serially diluted equimolar amount of DNA pooled from the target species (Figure 3). The relative ratio of normalized signals is shown in Table 2. A ratio value of 1 is thus an indication of similar amount of template or copy number. In a typical MLPA reaction on human DNA cut off values higher than 1.3 or lower than 0.7 are used to indicate copy number increase or gene duplication.
Multiplex ligation-dependent probe amplification and/or deletion. The normalized values show that at each concentration the relative signal intensity remains unchanged. The p value for the GLM-RM was >0.05, the standard deviations ranged between 0.007 – 0.09.

Figure 1. Electropherogram showing signals obtained from a mixed sample. Equimolar amounts of DNA pooled from the eight species used as template. Fragment sizes (nt) correspond to: 152 = selected streptococci, 171 = R. dentocariosa, 185 = Universal probe 1, 191 = C. albicans, 203 = A. gerencseriae, 237 = Universal probe 2, 252 = V. parvula, 301 = S. mutans, 326 = lactobacilli, 336 = A. naeslundii, 341 = L. acidophilus, 364 = A. odontolyticus. The universal probes target most of the oral bacteria.
Figure 2. Electropherogram showing single peaks generated by MLPA. Template DNA from individual species is used in the reaction. The MLPA reaction included all the 12 probes designed. Fragment sizes (nt) correspond to: 191 = C. albicans, 203 = A. gerencseriae, 336 = A. naeslundii, 364 = A. odontolyticus.
Figure 3. Relative quantification of MLPA fragments. Two fold serial dilution of DNA from each species and control plasmid (33 zmol) were used as template in each dilution series. Fluorescence data from control probes (141 nt and 237 nt) were used to normalize the data obtained from the samples. The reaction was done in triplicate. Intensity of the signal from each sample in a dilution series is divided by the sum of the signals from the control probes in the respective series. The resulting ratios were in turn divided by the average signal from the triplicate runs. The p value for the GLM-RM was >0.05, the standard deviations ranged between 0.007 – 0.09.
Table 2. Relative signal ratio of probes observed from peak area values. The values are normalized by dividing signal intensities of individual probes to the sum of signal intensities from all probes. The resulting ratio is again divided by the average of the triplicate values obtained from each probe. The p value for the GLM-RM was >0.05, the standard deviations ranged between 0.007 – 0.09.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fragment Size (nt)</th>
<th>Template DNA concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 ng</td>
<td>2 ng</td>
</tr>
<tr>
<td><em>R. dentocariosa</em></td>
<td>171</td>
<td>0.647</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>191</td>
<td>1.004</td>
</tr>
<tr>
<td><em>A. gerencseriae</em></td>
<td>203</td>
<td>1.016</td>
</tr>
<tr>
<td><em>V. parvula</em></td>
<td>252</td>
<td>0.981</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>301</td>
<td>0.998</td>
</tr>
<tr>
<td><em>A. naeslundii</em></td>
<td>336</td>
<td>1.024</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>341</td>
<td>1.010</td>
</tr>
<tr>
<td><em>A. odontolyticus</em></td>
<td>364</td>
<td>0.987</td>
</tr>
</tbody>
</table>

Application of MLPA on community DNA

With the selected set of MLPA probes, we observed that the MLPA profiles obtained from DNA isolated from different saliva donors were distinct. Moreover, the peak sizes, which indicate the relative abundance of detected bacteria in each sample, were also clearly different (data not shown). DNA obtained from CDF grown biofilm also showed a distinct pattern. The eight species in the mixed species biofilm or consortium that can be detected by our probes were traced back accurately. The strains abundance in the biofilm; as shown in fluctuation of their numbers due to changes in environment, incubation time and the type of media used was also apparent in the variable intensity of signals obtained (Figure 4). Microbial shift was discernible from the increase and decrease of signal intensities from all samples that were grown in different media. A microbial shift was also
observed in the biofilm samples grown in the CDFF (Figure 5). This shift was most pronounced in sucrose supplemented medium where a sharp increase in *A. odontolyticus* was observed. MLPA detected *S. mutans* in the CDFF samples grown in sucrose supplemented medium but not in the inoculum saliva and the medium without sucrose additive. Apparently, *S. mutans* was not present in detectable amount in this saliva sample. Changes were also evident in numbers of *R. dentocariosa* and *A. naeslundii*. These species were not detected anymore in the resulting biofilm samples. Reciprocally fluctuating streptococci and *Actinomyces* species in oral biofilms were reported in other studies as well (DALWAI et al. 2006; MOORE and MOORE 1994).

**Comparison of DGGE with MLPA**

In general, we found that the community compositions as deduced from DGGE profiles were in agreement with those from MLPA profiles. Relative band intensities of the samples whose products were visible on the gel corresponded to signal intensities obtained from MLPA profiles. However, the sensitivity of DGGE was lower than that of MLPA as judged by the observation that *A. gerencseriae* and *A. naeslundii* bands were not visible on the DGGE gel, but they were detected by MLPA analyses. In addition, DGGE seems to be more prone to PCR bias than MLPA. The fluctuation of *S. mutans* and *L. acidophilus* in the consortium was not detected by DGGE.
Figure 4. Variation in signal intensities of a biofilm (consortium) composed of eight species. The biofilm was grown in three different media for three different incubation times: (A) Biofilm grown in artificial saliva + sucrose + pipes medium; (B) Biofilm grown in artificial saliva + sucrose medium; (C) Biofilm grown in artificial saliva medium alone. Fragment sizes correspond to the following microorganisms: 191 = *C. albicans*, 203 = *A. gerencseriae*, 252 = *V. parvula*, 301 = *S. mutans*, 336 = *A. naeslundii*, 341 = *L. acidophilus*, 364 = *A. odontolyticus*. Signal ratios were obtained by dividing the signal intensity of each peak with the sum of all signals in the same series. These ratios were then divided by the respective average ratios of the triplicate runs. The p value for the GLM-RM was >0.05.
DISCUSSION

We designed a set of MLPA target sequences for the species we selected in this study, all of which yielded probe fragments of the expected size following the PCR based amplification method during MLPA. These probes tested on DNAs isolated from pure bacterial cultures, and communities contained in crude saliva as well as CDFF biofilms and consortium produced the expected specific signals with 100% accuracy. No cross reactivity was observed between probes. Variations in the MLPA profiles and peak areas obtained from different saliva donors confirmed that the
composition of the microflora in the saliva of each individual is unique and characteristic for the donor. The growth of *S. mutans* which is commonly implicated in cariogenicity is shown to be affected by sucrose supplement (Cury et al. 2001). The detection of this species in the sucrose supplemented experiment but not in the crude saliva or the artificial saliva medium seems to corroborate this. About 6,000 copies of target DNA is required in a standard MLPA reaction (Schouten et al. 2002). Pre amplification of target molecules followed by a proper dilution can be used to enhance the sensitivity of detecting targets that are found in very small amount (Reijans et al. 2008). MLPA is a PCR-based assay hence it is subject to PCR bias. We observed over saturation of the PCR reaction when a template is present in abundance, compromising the sensitivity of the assay for relative quantification.

The biofilms from the eight species consortium grown in Bijou tubes also show variation in incubation time and the medium used. The overall divergence between conditions varied from $10^{-40\%}$. This percentage indicates that a single parameter change could trigger dramatic changes in the composition of the microflora. This was more evident in our study particularly when the artificial saliva medium was supplemented with sucrose.

These results show the advantage of MLPA in investigating the dynamics of oral biofilm and accurate assessment of the spatial and temporal variations in the oral microbial communities. Understanding this dynamics and being able to monitor it would have a significant benefit in disease management (Liljemark et al. 1997; Marsh 1994). Fluctuations in composition and abundance of oral bacterial species were investigated using FISH (Al-Ahmad et al. 2007), quantitative real-time PCR (Lyons et al. 2000), Taq-man real-time PCR (Suzuki et al. 2005) and competitive PCR (Ruff et al. 1999). However, these studies focus on a few species and do not accurately reflect the situations in biofilms, albeit the species are considered to play important role in the onset of various dental problems. The simultaneous detection and relative quantification of up to 45 specific amplicons using MLPA provides an excellent opportunity to investigate the microbial dynamics as well as determining the presence and abundance of oral pathogens. Our results that showed an overall $2^{-7\%}$ divergence in relative signal ratio of amplified probes from normalized peak area values indicate that MLPA can be a cheaper alternative to real-time PCR.
assays. We compared the MLPA profiles obtained with the DGGE patterns and observed that MLPA is more sensitive. Comparison of MLPA with monoplex real-time PCR shows a comparable sensitivity (Reijans et al. 2008). Reducing the hybridization time to two hours did not have a significant effect on the sensitivity of the MLPA reaction. Therefore, the whole reaction can be performed within a working day. In addition, automation of the whole procedure and a high throughput detection of several samples in a single assay are feasible.

The data presented here show the applicability of MLPA in identification of bacterial species from a community. In general, this accurate multiplex assay can be used as a rapid diagnostic tool in identification of a pathogen and for monitoring microbial dynamics in complex bacterial communities. The advantage of MLPA lies in identifying up to 45 unique sequences in a single tube PCR reaction by using only a single primer pair. In addition, it is fast and easy to perform. Quantification of products where there is no large variation between copy numbers in samples is also possible. Comparison of MLPA to other widely used molecular methods such as FISH and STR confirmed that it is reliable, accurate fast and cost effective (Fernandez et al. 2005; Palomares et al. 2006). As was reported on Mycobacteria samples (Bergval et al. 2008), MLPA can also be used to screen clinical isolates and characterize pathogens. The 16S rDNA gene in bacteria contains about 1,500 nucleotides, of which conserved, variable and hypervariable regions span the whole molecule (Woese 1987). No other gene has been as extensively sequenced and studied for phylogenetic and taxonomic purposes as 16S rDNA. The outcome of the plethora of 16S rDNA based studies, a large sequence database (Maidak et al. 2001; Maidak et al. 1997), is often used for determining the taxonomic positions of both cultivable and un-cultivable bacterial strains. Bacterial genomes contain up to 15 copies of the 16S rDNA gene (Fogel et al. 1999). These different copies are shown to exhibit varying degree of redundancy and heterogeneity (Acinas et al. 2004). The variable regions of 16S rDNA are ideal for highly specific MLPA probe design. However, the small size of this gene presents a problem in designing an extensive MLPA-probe library especially from species or strains that have very similar sequences. Nevertheless, using unique MLPA probes targeting genus and species specific signature sequences in this molecule enable to identify bacterial species in a
community. Other phylogenetic marker sequences, such as from 23S rDNA (as used for *S. sanguinis* in this study) and other commonly used house-keeping genes, can also be used for MLPA probe design provided their specificity to the species can be determined by comparing with adequate number of closely related sequences.

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