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ORIGINAL ARTICLE

Analysis of interspecies adherence of oral bacteria using a membrane binding assay coupled with polymerase chain reaction-denaturing gradient gel electrophoresis profiling

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Information on co-adherence of different oral bacterial species is important for understanding interspecies interactions within oral microbial community. Current knowledge on this topic is heavily based on pariwise coaggregation of known, cultivable species. In this study, we employed a membrane binding assay coupled with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to systematically analyze the co-adherence profiles of oral bacterial species, and achieved a more profound knowledge beyond pairwise coaggregation. Two oral bacterial species were selected to serve as "bait": *Fusobacterium nucleatum* (*F. nucleatum*) whose ability to adhere to a multitude of oral bacterial species has been extensively studied for pairwise interactions and *Streptococcus mutans* (*S. mutans*) whose interacting partners are largely unknown. To enable screening of interacting partner species within bacterial mixtures, cells of the "bait" oral bacterium were immobilized on nitrocellulose membranes which were washed and blocked to prevent unspecific binding. The "prey" bacterial mixtures (including known species or natural saliva samples) were added, unbound cells were washed off after the incubation period and the remaining cells were eluted using 0.2 mol $Ł^{-1}$ glycine. Genomic DNA was extracted, subjected to 16S rRNA PCR amplification and separation of the resulting PCR products by DGGE. Selected bands were recovered from the gel, sequenced and identified *via* Nucleotide BLAST searches against different databases. While few bacterial species bound to *S. mutans*, consistent with previous findings *F. nucleatum* adhered to a variety of bacterial species including uncultivable and uncharacterized ones. This new approach can more effectively analyze the co-adherence profiles of oral bacteria, and could facilitate the systematic study of interbacterial binding of oral microbial species.

Keywords: membrane binding assay; polymerase chain reaction-denaturing gradient gel electrophoresis; coaggregation; *Fusobacterium nucleatum*; *Streptococcus mutans*

Introduction

More than 600 bacterial species inhabit the human oral

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cavity [1-3] in highly organized communities commonly known as dental plaque. These dental plaque communities [4] are responsible for many oral infectious diseases including dental caries and periodontal disease [5-8]. Extensive studies have revealed many of the complex interactions between the residents of the oral microbial community [9-10]. During dental plaque formation, the "early colonizing" bacteria express components that

enable effective adherence to the target tissues including teeth surface and mucosa [11-12]. Late colonizers adhere to the early colonizers or "bridging organisms" such as *Fusobacterium nucleatum* (*F. nucleatum*) to integrate into the community [13]. The ability to adhere to other bacteria species is a fairly common characteristic among oral microbes. It has been implicated to play an important role in the formation of structured multispecies oral biofilms that affect the microbial ecology and pathology of oral cavity [14-15].

Most investigations of oral bacterial interspecies binding have relied on a coaggregation assay developed by Cisar *et al.* more than 30 years ago in which pairwise combinations of bacteria are tested in planktonic culture [14, 16-18]. A more recent membrane-based binding assay developed by Lamont *et al.* increased the sensitivity of detecting interbacterial binding by immobilizing one bacterial species on a membrane and using radio-labeled test organism for easy detection and quantification [9]. In this study, we expanded from this previously developed membrane binding assay which was limited to examining one-on-one interactions and used complex salivary samples cultured in the recently reported SHI medium that supports growth of diverse oral communities [19] for screening against the membrane bound "bait" organism. Subsequent polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiling and species identification allowed investigation of the comprehensive co-adherence profiles of *F. nucleatum* and *Streptococcus mutans* (*S. mutans*). We demonstrated that compared with existing assays for studying bacterial co-adherence, this new approach is effective in simultaneous identification of multiple co-adherence partners. Furthermore, the increased species diversity achieved by using SHI medium for cultivating oral microbial flora from saliva can be used to discover new co-adherence partners including previously uncul-turable bacteria.

Materials and Methods

Bacteria and culture conditions

Bacterial strains used in this study include *S. mutans* UA140, *Porphyromonas gingivalis* (*P. gingivalis*) W83, *F. nucleatum* ATCC23726, *Streptococcus mitis* (*S. mitis*) ATCC9811, *Escherichia coli* (*E. coli*) DH5α. *S. mutans*, *S. mitis* and *E. coli* were grown in Brain Heart Infusion (BHI, Difco) medium. *F. nucleatum* and *P. gingivalis* were grown in Columbia Broth (CB, Difco). *E. coli* was grown under aerobic condition at 37 ℃, while all other bacterial strains were grown in an anaerobic atmosphere

of 80% N_2, 10% CO_2, and 10% H_2 at 37 ℃.

Saliva collection

Saliva samples were collected from six healthy subjects, age 25–35 years under UCLA-IRB #09-08-068-02A. Subjects with systemic diseases or taking prescription or non-prescription medication were excluded from the study. Subjects were asked to refrain from any food or drink 2 hours before donating saliva and to spit directly into the saliva collection tube, 5 mL saliva was collected from each person. Saliva samples were pooled and centrifuged at 2 600×*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 planktonic culture in SHI medium [19].

Mice gut microbial samples

The mice gut microbial samples were recovered from a lab stock that was described in a previous study [20].

Membrane binding assay

A membrane binding assay was developed to detect the adherence of salivary bacteria to selected oral microbes (bait organisms) immobilized on a solid support according to a previously described assay [9] with some modifications. Nitrocellulose membranes (0.45 μm pore size; GE Water and Process Tech) were cut into 4 cm^2 pieces and each piece was placed in a sterile Petri dish (100 mm × 15 mm, Fisher, USA). Bait organisms were harvested by centrifugation at 10 000×*g* for 10 min. Cell pellets were washed twice and resuspended in PBS buffer. The cell concentration was adjusted to $2×10^9$ cells·mL^{-1} and 50 μL of the cell suspension was added to the centre of each nitrocellulose membrane. The nitrocellulose with immobilized bacteria (bait organism blot) was washed three times (15 min each) in PBS buffer containing 0.1% Tween 20 (PBST) to remove loosely bound organisms. The non-specific sites of the nitrocellulose were blocked by soaking in PBST with 5% BSA, 5% non-fat dry milk and 200 μg·mL^{-1} fish sperm DNA. The membrane was further washed 3 times with PBS buffer (15 min each).

The test bacteria were harvested by centrifugation at 10 000×*g* for 10 min, washed twice and resuspended in PBS buffer to a final concentration of 10^7 cells·mL^{-1}. Defined bacterial mixture was constructed by mixing at

equal culture volumes different combinations containing three of the following four species: *F. nucleatum*, *S. mitis*, *P. gingivalis* or *E. coli*. 10 mL of the bacterial mixture was added into each Petri dish containing the nitrocellulose membrane with bait organism. After incubation for 2.5 h at 37 ℃ on a rotary shaker at 20 tilts·min^{-1}, unbound cells were decanted, and the blot was washed four times for 15 min each with PBST. The areas of the nitrocellulose containing the bait species were excised, the bound organisms were washed off by 0.2 mol·L^{-1} glycine (pH=2.7) and collected. Nitrocellulose membrane without base organism blot was used as blank control.

PCR-DGGE analysis

Total genomic DNA of bacteria was isolated using the QIAamp^{TM} DNA Micro Kit (QIAGEN, USA). DNA quality and quantity were measured by a UV spectrophotometer at 260 nm and 280 nm (Spectronic Genesys^{TM}, Spectronic Instrument, Inc., Rochester, NY).

Bacterial 16S ribosomal RNA (rRNA) genes were amplified by PCR as described previously [21]. In brief, the universal primer set, Bac1 (5'-CGCCCGCCGCGC CCCGCGCCCGTCCCGCCGCCCCCGCCCGACTAC GTGCCAGCAGCC-3') [22] and Bac2 (5'-GGACTACC AGGGTATCTAATCC-3') was used to amplify an approximately 300 base pair (bp) internal fragment of the 16S rRNA gene. Each 50 µL PCR contained 100 ng purified genomic DNA, 40 pmol of each primer, 200 µmol·L^{-1} dNTPs, 4 mmol·L^{-1} MgCl$_2$, 5 µL 10× PCR buffer, and 2.5 U Taq DNA polymerase (Invitrogen). Cycling conditions were 94 ℃ for 3 min, followed by 30 cycles of 94 ℃ for 1 min, 56 ℃ for 1 min and 72 ℃ for 2 min, with a final extension period of 5 min at 72 ℃. The resulting PCR products were evaluated by electrophoresis in 1% agarose gels.

Polyacrylamide gels at an 8% concentration were prepared with a denaturing urea/formamide gradient between 40% [containing 2.8 mol·L^{-1} urea and 16% (*V/V*) formamide] and 60% [containing 4.2 mol·L^{-1} urea and 24% (*V/V*) formamide]. Approximately 300 ng of the PCR product was applied per well. The gels were submerged in 1× TAE buffer (40 mmol·L^{-1} Tris base, 40 mmol·L^{-1} glacial acetic acid, 1 mmol·L^{-1} ethylenediaminetetraacetic acid) and the PCR products were separated by electrophoresis for 17 h at 58 ℃ 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 destaining in 1× TAE buffer. DGGE profile images were digitally recorded using the Molecular Imager Gel Documentation system

(Bio-Rad Laboratories).

Sequencing of PCR products

PCR products were excised from the DGGE gel, eluted into 20 µL sterile dH$_2$O as preciously described [23] and re-amplified with the Bac1/Bac2 universal primers. The resulting PCR products were purified and sequenced at the UCLA sequencing and genotyping core facility.

Sequence analysis

The obtained partial 16S rRNA gene sequences were used to BLAST search the HOMD (http://www.homd.org) and NCBI (http://www.ncbi.nlm.nih.gov) databases. Sequences with 98% to 100% identity to those deposited in the public domain databases were considered to be positive identification of taxa.

Coaggregation assay

Coaggregation assays were performed to determine the interbacterial binding in suspension as previously described [18]. In brief, bacteria were collected and resuspended in coaggregation buffer containing 150 mmol·L^{-1} NaCl, 1 mmol·L^{-1} Tris/HCl pH 8, 0.1 mmol·L^{-1} CaCl$_2$, 0.02% NaN$_3$, and 0.1 mmol·L^{-1} MgCl$_2$ [14]. The concentration of the bacterial suspensions was adjusted to 2×10^9 cells·mL^{-1} and equal volumes (0.2 mL) were mixed in sterile test tubes by vortexing for 30 s (Vortex-2 genie, Scientific industries, USA), followed by gentle agitation for 10 s. After allowing the mixtures to settle for 30 s, the degree of coaggregation was scored using a semi-quantitative assay originally described by Cisar *et al.* [14]. Tubes containing: (a) coaggregation buffer solution alone; and (b) each test bacterial suspension alone, served as negative control.

Results

Membrane binding assay allowed identification of multiple co-adherence partners for single bacterial species from a defined microbial community

For comparison with the classical coaggregation assay, defined microbial communities containing known bacterial species were used to test the reliability and reproducibility of the membrane-based assay used in this study.
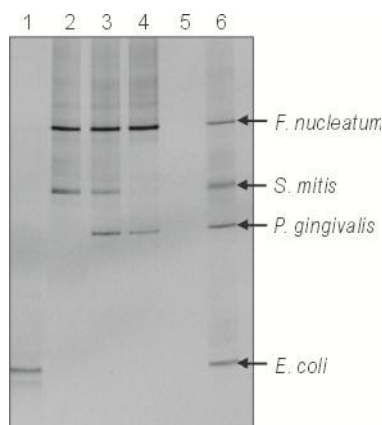
The coaggregation profiles of *F. nucleatum*, *S. mitis*, *P. ginigivalis* and *E. coli* with the strains used in this study were tested with the standard coaggregation assay (Table 1). Each species was then used as the "bait" organism in the membrane assay and incubated with the corresponding mixtures of the remaining species. PCR-DGGE analysis revealed that the membrane assay

reflected the results of the coaggregation assay (Figure 1). When *F. nucleatum* was used as bait organism, both *S. mitis* and *P. gingivalis* were bound, while *S. mitis* and *P. gingivalis* only interacted with *F. nucleatum*. *E. coli* was unable to interact with any of the oral species present in the defined community.

Previous study indicated that coaggregation of *F. nucleatum* with certain oral bacterial species could be inhibited by arginine [24]. Our coaggregation assay confirmed this observation, showing that the presence of 50 mmol·L^{-1} arginine completely abolished the coaggregation between *F. nucleatum* and the test organisms (Table 2). This was also accurately reflected by the membrane binding assay, which showed that in the presence of arginine, bands corresponding to *S. mitis* and *P. gingivalis* were no longer observed in the DGGE profile of the interacting partner species of *F. nucleatum* (Figure 2).

Table 1 Coaggregation properties of *F. nucleatum*, *S. mitis*, *P. gingivalis* and *E. coli*

| species | Coaggregation score* with | | | |
	F. nucleatum	*S. mitis*	*P. gingivalis*	*E. coli*
F. nucleatum	0			
S. mitis	4	0		
P. gingivalis	3	0	0	
E. coli	0	0	0	0

*0: no visible coaggregation; 1: small coaggregates, remaining in solution; 2: larger coaggregates, not immediately falling out of solution; 3: larger coaggregates, immediately clearing, slightly turbid suspension; 4: large coaggregates, setting immediately.
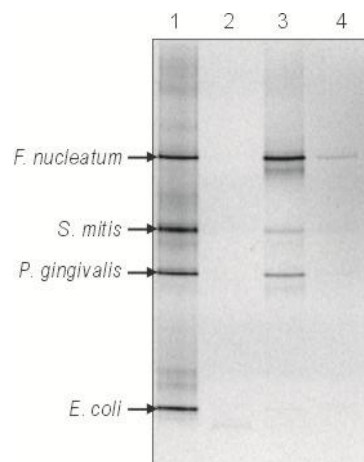
Table 2 Coaggregation of *F. nucleatum with S. mitis, P. gingivalis* and *E. coli* in the presence/absence of arginine

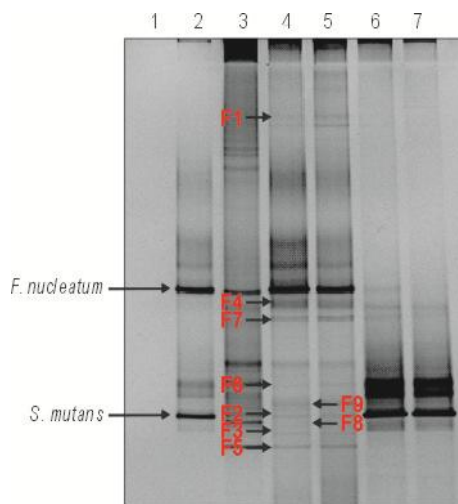| species | Coaggregation score* with | | | |
	F. nucleatum	*S. mitis*	*P. gingivalis*	*E. coli*
F. nucleatum Without arginine	0	4	3	0
F. nucleatum With arginine**	0	0	0	0

* see legend in table 1 for details. ** 50 mmol·L^{-1} arginine.

Figure 1 Co-adherence profiles of selected species with a defined bacterial mixture. PCR-DGGE analysis showiing lane 1: *E. coli* as bait organism incubated with a mixture containing *F. nucleatum, S. mitis* and *P. gingivalis*; lane 2: *S. mitis* as bait organism incubated with a mixture containing *F. nucleatum, E. coli* and *P. gingivalis*; lane 3: *F. nucleatu*m as bait organism incubated with a mixture containing *S. mitis, E. coli* and *P. gingivalis*; lane 4: *P. gingivalis* as bait organism incubated with a mixture containing *F. nucleatum*, *E. coli* and *S. mitis*; lane 5: control nitrocellulose membrane without bait organism incubated with experimental organisms; lane 6: ladder containing *F. nucleatum*, *S. mitis*, *P. gingivalis* and *E. coli*. Two biological replicates were performed and a representative gel image is shown.

Figure 2 Co-adherence profiles of *F. nucleatum* with a defined bacterial mixture in the presence and absence of arginine. PCR-DGGE analysis showing lane 1: ladder containing *F. nucleatum*, *S. mitis*, *P. gingivalis* and *E. coli*; lane 2: control nitrocellulose membrane without bait organism incubated with experimental organisms; lane 3: *F. nucleatum* as bait organism incubated with a bacterial mixture containing *S. mitis, E. coli* and *P. gingivalis* in the absence of arginine; lane 4: *F. nucleatum* as bait organism incubated with a mixture containing *S. mitis, E. coli* and *P. gingivalis* in the presence of 50 mmol·L^{-1} arginine. Two biological replicates were performed and a representative gel image is shown.

Analysis of the co-adherence profiles of selected oral bacteria

To further test the effectiveness of the new approach in identifying the co-adherence partners from a complex microbial community, we chose *F. nucleatum* and *S. mutans*, two clinically relevant oral bacterial species, as the bait organisms to identify their co-adherence partners from salivary microbial flora cultivated in SHI medium. The membrane binding assay revealed that these two selected oral bacterial species displayed distinct co-adherence profiles with salivary bacteria (Figure 3). The co-adherence partners of *S. mutans* were limited to *Fusobacterium* sp., *Haemophilus* sp. and *Streptococcus* sp., while *F. nucleatum* adhered to a variety of bacterial species including *Fusobacterium* sp., *Gemella* sp., *Granulicatella* sp., *Haemophilus* sp., *Neisseria* sp., *Peptostreptococcus* sp., *Porphyromonas* sp., *Prevotella* sp., and *Streptococcus* sp.. These data were consistent with previously reported coaggregation assay result [25-26]. More importantly, our assay identified several previously unculturable species that adhered to *F. nucleatum* in the membrane binding assay.

Oral bacterial species displayed less co-adherence with mice gut bacteria

Previous studies employing the standard coaggregation assay have shown that bacteria from different microbial communities exhibit less interbacterial binding than species originating from the same community [27]. Therefore, we investigated the co-adherence profiles of *F. nucleatum* and *S. mutans* with a foreign flora using cultivated mice gut flora as a model community. Neither oral bacterial species exhibited significant co-adherence with mice gut bacteria. The only gut species found to bind to *F. nucleatum* was identified as *Lactobacillus* sp. (Figure 4).
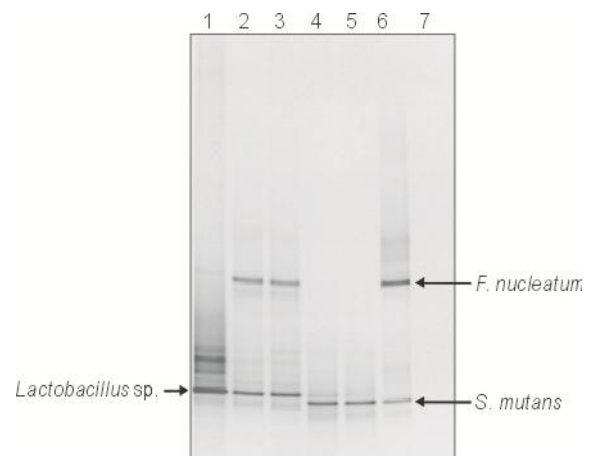

Figure 3 Co-adherence profiles of *F. nucleatum* and *S. mutans* with SHI medium cultured salivary microbial community. PCR-DGGE analysis showing lane 1: control nitrocellulose membrane without bait organism incubated with experimental organisms; lane 2: ladder of bait organisms *F. nucleatum* and *S. mutans*; lane 3: SHI medium cultured salivary bacteria; lane 4 and 5: two independent replicates of *F. nucleatum* as bait organism incubated with SHI medium cultivated salivary bacteria; lane 6 and 7: two independent replicates of *S. mutans* as bait organism incubated with SHI medium cultivated salivary bacteria. Sequencing results are indicated: F1-*Fusobacterium* sp.; F2-*Gemella* sp.; F3-*Granulicatella* sp.; F4-*Neisseria* sp.; F5-*Peptostreptococcus* sp.; F6-*Porphyromonas* sp.; F7, F8, F9-unclassified uncultured bacteria.

Figure 4 Co-adherence profiles of *F. nucleatum* and *S. mutans* with BHI cultivated mice fecal bacteria. PCR-DGGE analysis showing lane 1: BHI cultured mice fecal bacteria; lane 2 and 3: two independent replicates of *F. nucleatum* as bait organism incubated with BHI cultivated mice fecal bacteria; lane 4 and 5: two independent replicates of *S. mutans* as bait organism incubated with BHI cultivated mice fecal bacteria; lane 6: ladder of bait organisms *F. nucleatum* and *S. mutans*; lane 7: control nitrocellulose membrane without bait organism incubated with experimental organisms.

Discussion

The bacterial residents within oral microbial community display extensive interactions with each other [10].

The physical binding between two oral bacterial species in suspension was first documented in 1970 [15]. It is thought to be a highly specific recognition and adhesion between surface components of different bacterial species. This interaction has also been suggested to play a pivotal role in establishing the highly structured multispecies oral plaque communities [28-29]. The coaggregation assay in which two test microbes were mixed in suspension and the interbacterial interaction was determined by monitoring the precipitation as a result of formation of coaggregates, has been the standard assay to investigate the physical interaction between different bacterial interspecies [30-31]. By immobilizing one bacterial species on a nitrocellulose membrane as "bait" organism and adding radio-labeled testing species, Lamont *et al*. developed a membrane-based binding assay to measure the binding between two bacterial species [9]. Due to its higher sensitivity, this new assay revealed novel interbacterial binding that could not be shown by the standard coaggregation assay. However, like other methods, this membrane assay is limited to bacterial species that can be grown in culture, and only examines bacterial co-adhesion at a dual species level. Since over 600 bacterial species reside in the human oral cavity [3], the current approaches of investigating bacterial interaction one-on-one makes the systematic study of interbacterial binding of oral bacteria a difficult task. Furthermore, more than 50% of the oral microflora are unculturable and cannot to be characterized using these types of assays [32].

To overcome these limitations, we developed a new approach by combining the membrane binding assay with PCR-DGGE profiling. Nitrocellulose membranes were used for immobilizing "bait" organisms, which has been shown to provide ease of manipulation for studying interspecies co-adhesion [9, 33], while the addition of PCR-DGGE enhanced the analyzing capacity. As a community-based molecular technique for analyzing microbial community profiles, PCR-DGGE is capable of detecting multiple bacterial species within the same microbial sample [10]. Our data derived from defined bacterial mixtures demonstrated that the newly developed approach was able to identify co-adherence partners which were verified by the classical coaggregation assay (Table 1 and Figure 1). The addition of arginine resulted in the same outcome for both assays (Table 2 and Figure 2), indicating that similar recognition and adhesion mechanisms are involved in the interbacterial binding under the different experimental setups.

F. nucleatum and *S. mutans* were selected as oral representative bacterial species to study their co-adherence profiles within a salivary microbial community in more detail. Due to its ability to coaggregate with a variety of oral bacterial species, *F. nucleatum* is considered a "bridging organism" and has been suggested to play an important role in the development of oral microbial communities [4, 8, 34], while *S. mutans* is one of the early colonizers which can adhere to teeth surface by producing glucans and is generally regarded as a cariogenic bacterium [35-37]. We hypothesized that considering the different roles of these two bacterial species in dental plaque formation they could possess different co-adherence ability and have distinct interacting partners. Our data revealed that these two bacterial species indeed displayed different co-adherence profiles, with *F. nucleatum* showing a far more diverse panel of interacting partner (Figure 3) which is consistent with its role as a bridging organism in biofilm architecture. More importantly, the coupling of the membrane binding assay with PCR-DGGE profiling enhanced the identification capacity of co-adherence partners from a complex microbial community and allowed simultaneous detection of multiple bacterial interacting partners of *F. nucleatum* in the same experiment (Figure 3). Furthermore, by using the recently developed SHI medium, which is much less selective than other media in cultivating oral microbes [19], we were able to broaden the spectrum of microorganisms that adhere to *F. nucleatum* by discovering interactions with currently unculturable and uncharacterized bacteria.

Previous studies showed that bacteria from different origins were less likely to have direct physical binding as revealed by coaggregation assay [27]. Using BHI cultivated mice gut microbial community [20] we further tested the interbacterial binding between oral species and gut flora. Our data are in agreement with the previous reports, showing that the only mice gut bacterial species displaying binding capability with *F. nucleatum* was *Lactobacillus* sp. (Figure 4). Lactobacilli belong to the few gastrointestinal tract associated bacteria that are found in both oral cavity and intestinal tract of human and mice.

Conclusion

The new approach developed in this study combines the advantages of the membrane binding assay and PCR-DGGE profiling technique and allows for efficient analysis of co-adherence profiles of oral bacterial species. Our data demonstrated its effectiveness in identifying multiple co-adherence partners from complex microbial community and will greatly enhance our ability to systematically study the interbacterial binding within the community. Furthermore, this assay could be expanded

to search for binding partners within original environmental samples, such as saliva, to identify unculturable bacterial species and expand our knowledge regarding bacterial interactions.

Acknowledgement

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