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The influence of iron availability on human salivary microbial community composition

Renke Wang^{2,#}, Aida Kaplan^{1,#}, Lihong Guo¹, Wenyuan Shi¹, Xuedong Zhou², Renate Lux¹, and Xuesong He^{1,*}

¹UCLA School of Dentistry, Los Angeles, CA 90095, USA

²State Key Laboratory of Oral Diseases, West China School of Stomatology, Sichuan University, Chengdu 610041, China

Abstract

It is a well-recognized fact that the composition of human salivary microbial community is greatly affected by its nutritional environment. However, most studies are currently focused on major carbon or nitrogen sources with limited attention to trace elements like essential mineral ions. In this study, we examined the effect of iron availability on the bacterial profiles of an *in vitro* human salivary microbial community as iron is an essential trace element for the survival and proliferation of virtually all microorganisms. Analysis via a combination of PCR with denaturing gradient gel electrophoresis (DGGE) demonstrated a drastic change in species composition of an *in vitro* human salivary microbiota when iron was scavenged from the culture medium by addition of the iron chelator 2,2'- bipyridyl (Bipy). This shift in community profile was prevented by the presence of excessive ferrous iron (Fe²⁺). Most interestingly, under iron deficiency, the *in vitro* grown salivary microbial community became dominated by several hemolytic bacterial species, including *Streptococcus* spp., *Gemella* spp. and *Granulicatella* spp.all of which have been implicated in infective endocarditis. These data provide evidence that iron availability can modulate host-associated oral microbial communities, resulting in a microbiota with potential clinical impact.

Keywords

iron availability; microbial flora; oral cavity

INTRODUCTION

Microorganisms require a variety of trace elements for their normal metabolic activities (1–2). One of the most essential trace elements for virtually all bacteria is iron which participates in many central biological processes, such as respiration, the tricarboxylic acid (TCA) cycle, oxygen transport, photosynthesis and DNA biosynthesis(1). The bacterial uptake of iron includes its physiologically relevant species, Fe²⁺ (ferrous iron) and Fe³⁺ (ferric iron). While Fe²⁺ is soluble under anaerobic condition and can be transported non-specifically by bacterial divalent metal ABC transporters, Fe³⁺ (the most predominant form of iron in natural environments) is not readily bio-available due to its hydrolysis and polymerization into insoluble forms (1, 3). The extremely low solubility (10⁻¹⁸ M at pH7) of Fe³⁺ makes iron one of the growth limiting factors within many ecological niches (1, 3–5).

*Corresponding author. UCLA School of Dentistry, 10833 Le Conte Avenue, CHS 20-118 Los Angeles, CA 90095-1668. Phone: (310) 825-9748. FAX: (310) 794-7109. xhe@ucla.edu.

#These authors contribute equally to the work.

This is particularly true for microbial niches within the human host where availability of iron to host-associated microbes on mucosal surfaces is further restricted due to sequestration by host iron-binding proteins (6–8). As an effective host defensive mechanism, human body maintains an iron-restricted environment where most extracellular iron is bound to iron-withholding glycoproteins such as transferrin and lactoferrin, while intracellular iron is sequestered by heme or ferritin compounds (6–7, 9). The concentration of free iron in living tissues has been estimated to be only 10^{-18} M, far below the concentration required for bacterial growth (7).

The oral cavity is home to several hundred different species of bacteria (10–13). These microorganisms form an organized multispecies community with intricate interactions which include competing for nutrients between the biofilm residents (14–15). The sources of nutrients for oral microbial flora include saliva, crevicular fluid and host diet. Saliva which is considered the main nutrient source due to its chemical composition and continuous production (16) has a very limited supply of free iron with most of the iron bound to lactoferrin, a host-produced transferrin protein (17–18). To cope with the limited availability of free iron within the oral cavity, the resident bacteria employ various mechanisms to close the gap between Fe^{3+} solubility and their iron requirements, by either reducing Fe^{3+} to the soluble Fe^{2+} via a membrane associated ferric reductase activity (19), producing siderophores as high-affinity ferric chelators under iron-limiting conditions (20–21), directly acquiring and utilizing human transferrin (22), or acquiring iron by importing heme via heme-binding protein (Hbp), e. g. HmuR in *Porphyromonas gingivalis* (23). The virtually universal demands for iron and its limited bio-availability, together with the differential capabilities of microbes to acquire iron and persist under iron-deficient conditions, are likely to make iron one of the determining nutritional factors in modulating the composition of oral microbiota. In this study we sought to investigate the effect of iron availability on the overall microbial profiles of *in vitro* human salivary bacterial communities, and characterize the dominant bacterial species selected under free iron-deficient condition.

MATERIALS AND METHODS

Saliva collection

Saliva samples were collected from six healthy subjects, age 25–35 under UCLA-IRB #09-08-068-02A. None have been taking any prescription or non-prescription medications or being treated for any systemic disease. Subjects were asked to refrain from any food or drink 2 hrs before donating saliva. 5 ml un-stimulated saliva was obtained from each subject by having them spit directly into a saliva collection tube. 2.5 ml of each individual saliva sample was pooled together and referred to as pooled saliva; while the rest of each saliva sample was referred to as individual saliva and used throughout this study.

Culturing saliva-derived microbial flora and bacterial isolation

200 μl of pooled or individual saliva was inoculated into 2 ml of SHI medium(24) or artificial saliva solution (ASS) defined medium (25). Different concentrations of the iron chelator, 2,2'-bipyridyl (Bipy) (Fisher Scientific, NJ) and/or Fe^{2+} (Fe^{2+} solution was made fresh by dissolving FeSO_4 in reduced ddH₂O, followed by filter sterilization) were added to the media prior to inoculation of the saliva samples. Cultures were incubated under anaerobic conditions (85% nitrogen, 5% carbon dioxide and 10% hydrogen) for 18 hrs at 37°C. 1 ml of bacterial sample from each culture was taken and bacterial cells were collected by centrifugation at 14,000 x g for 3 min and used for total DNA extraction.

For isolation of individual bacterial species, the OD₆₀₀ of the overnight culture was adjusted to 0.1, followed by serial dilution with PBS buffer and plating onto SHI medium agar plates.

The plates were incubated under anaerobic conditions for 3 days at 37°C to allow development of single colonies. Based on their difference in color and morphology, colonies were picked and grown in SHI medium. Cells from each isolate were collected, stocks were made in SHI medium containing 25% glycerol and stored at -80°C for further studies.

PCR-DGGE analysis

Total genomic DNA of bacterial samples was isolated using the MasterPure™ DNA Purification Kit (EPICENTRE), DNA quality and quantity were measured by a spectrophotometer at 260nm and 280nm (Spectronic Genesys™, Spectronic Instruments, Inc. Rochester, NY). Amplification of bacterial 16S ribosomal RNA genes by PCR was carried out as described previously (26). 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') (27) 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 of each dNTP, 4.0 mM MgCl₂, 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 1 min, with a final extension period of 5 min at 72°C. The resulting PCR products were evaluated by 1% agarose gel electrophoresis.

Polyacrylamide gels at an 8% concentration were prepared with a denaturing urea/formamide gradient between 40% [containing 2.8 M urea and 16% (vol/vol) formamide] and 70% [containing 4.9 M urea and 28% (vol/vol) 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 hrs at 58°C using a fixed voltage of 60 V in the Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, gels were rinsed and stained for 15 min in 1× TAE buffer containing 0.5 µg/ml 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).

Identification of bacterial isolates and major bacterial species within salivary microbial communities with different treatments

For identification of bacterial isolates, the universal bacterial 16S rDNA primer pair, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC C TT GTT ACG ACT T-3'), was used to generate an approximately 1,500-bp amplicon. Each 50 µl PCR reaction mixture contained 20 ng of genomic DNA, 200 µM of each dNTP, 4.0 mM MgCl₂, 100 nM of each primer, 5 µl of 10× PCR buffer, and 2.5 U of Taq polymerase (Invitrogen). PCR conditions were as follows: 3 min at 94°C for initial denaturation and 27 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min and a final chain elongation at 72°C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen).

To identify major bacterial species within salivary communities, PCR bands were excised from the DGGE gel, eluted into 20 µl sterile dH₂O as previously described (28) 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. The obtained partial 16S rRNA gene sequences, as well as the 1,500-bp amplicons for bacterial single isolates, were used to BLAST search against 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.

Hemolysis assay

For examination of the hemolytic phenotype, salivary microbial communities or individual isolates grown in SHI medium supplemented with or without iron chelator were harvested and serially diluted (10-fold) from 10^0 to 10^{-4} . 10 μ l of each dilution from each sample was spotted onto SHI medium agar plates and grown for 3 days under anaerobic conditions at 37°C. Hemolytic activity was determined as follows: α -hemolysis presents with darkening and greenish coloration of the agar under the colonies; β -hemolysis shows lightened and transparent circles under the colonies; while lack of hemolytic activity, also known as γ -hemolysis results in no change of the agar coloration (29).

RESULTS

The iron chelating compound 2, 2'-bipyridyl (Bipy) induced a shift in bacterial profile of salivary microbial community cultivated in SHI medium

The effect of iron availability on the composition of an oral bacterial community was investigated by titrating free iron in the bacterial growth medium via the addition of the iron chelator 2, 2'-bipyridyl (Bipy). Human pooled saliva was inoculated into SHI medium containing 5% sheep blood which was previously described as being able to sustain the growth of a diverse *in vitro* microbial community similar to the original salivary microbiota (24) and a panel of increasing Bipy concentrations was added to remove free iron from the medium. PCR-DGGE analysis revealed a distinct Bipy-induced shift in the overall microbial profile of the salivary community after overnight growth (Fig. 1). In the absence of Bipy, the microbial community was comprised of a variety of bacterial species, including *Fusobacterium periodonticum*, *Neisseria subflava*, *Porphyromonas* spp., *Campylobacter* spp. and *Streptococcus* spp., while the addition of the iron chelator induced a pronounced shift in the community composition at an apparent threshold value of >0.2 mM with *Streptococcus* spp., *Gemella* spp. and *Granulicatella* spp. becoming the dominant species (Fig.1).

To rule out the possibility that the observed Bipy-induced community shift was due to toxic effects of the compound rather than lowering the free iron concentration in the medium, Bipy was added to cultures supplemented with 0.1 mM free iron (Fe^{2+}) and compared to unsupplemented cultures (Fig. 2). Consistent with our previous observation (Fig. 1) 0.3 mM Bipy alone caused a drastic community shift. However, addition of 0.1 mM (Bipy binds Fe^{2+} in a 3 to 1 stoichiometry) free iron ions (Fe^{2+}) eliminated the Bipy-induced community shift and the microbial profile remained similar to the original sample. The addition of 0.1 mM Fe^{2+} alone did not have a significant effect on community composition. Taken together these results confirmed that the observed bacterial community shift was indeed due to Bipy-based reduction in the iron concentration in the medium and not other effects of the chelating compound.

Bipy induced comparable profile shifts in salivary microbial flora isolated from different healthy human subjects

Next, we investigated if the Bipy-induced shift in the salivary microbial community composition is a general phenomenon by examining its effect on the individual salivary microbiota collected from different healthy subjects. Community shifts similar to the one revealed for pooled saliva was observed in all individual samples (Fig. 3). More importantly, all the shifts displayed a similar trend, with *Streptococcus* spp., *Granulicatella* spp. and *Gemella* spp. being the most prevalent bacteria in the presence of high concentration of iron chelator. Interestingly, for the saliva sample from subject 3, the addition of Bipy also

resulted in an increase in the population of *Abiotrophia defectiva*, often known as nutritionally variant streptococcus (NVS) or *Streptococcus defectivus*, a causative agent of infective endocarditis.

The availability of free iron ions (Fe^{2+}) modulated the microbial profile of salivary bacterial community

To further investigate the modulating effect of availability of free iron on the salivary microbial community, we generated a mixed microbial community containing bacterial populations cultivated from iron limiting as well as non-iron-limiting conditions. The resulting community with a mixed bacterial profile was then re-cultivated under either iron-depleted or repleted condition, and the modulating effect of iron accessibility on the microbial population profile was evaluated by monitoring the microbial population shift. Specifically, we inoculated pooled saliva samples in SHI medium in the absence and presence of Bipy at a final concentration of 0.3 mM which has been shown to induce a significant shift in community composition (Fig. 1) to generate culture-A and culture-B, respectively. After overnight incubation, bacterial cells in these two cultures were collected, resuspended in fresh SHI medium to an $\text{OD}_{600\text{nm}}$ of 0.2 and mixed in a 1:1 ratio to obtain co-culture (A+B). Co-cultures were supplemented either with Bipy or Fe^{2+} ions and incubated further for 16 hrs under anaerobic condition. PCR-DGGE analysis revealed that addition of Bipy to the microbial population present in the combined culture induced a shift in the community profile that resembled the Bipy-treated original sample. Exogenously provided Fe^{2+} in contrast modulated the mixed population resulting in a profile that was similar to the original untreated community (Fig. 4).

Bacterial species isolated from medium deficient in free iron generally displayed high hemolytic activity

One of the important components in SHI medium is sheep blood. It has been reported that certain oral bacterial species, such as *Streptococci* are able to lyse red blood cells, retrieve and utilize iron from heme to sustain their growth (20, 30–31). We speculated that the dominant species selected under high iron chelator concentrations in SHI medium could employ a similar strategy to obtain iron. To test this, we cultivated saliva samples in the presence and absence of Bipy. The overnight cultures were harvested and spotted onto blood agar plates in serial dilutions. The bacterial community selected in the presence of the iron chelator exhibited distinct α -hemolytic activity, while cells harvested from cultures supplemented with iron displayed no obvious hemolytic activity, similar to those cultivated in original SHI medium (Fig 5A). These results suggested that oral bacterial species selected under low free iron conditions are better adapted to obtain iron from their host environments. In order to further investigate this possibility, we chose three different *Streptococci* strains and one *Gemella* strain that were able to grow in the presence of high concentration of Bipy and tested their hemolytic activity. All four tested strains displayed various degree of α -hemolytic activity (Fig.5B, C); while species isolated from the original SHI medium culture, such as *F. nucleatum*, did not show significant hemolytic activity under the conditions tested. More intriguingly, some of the isolated strains, including *Gemella haemolysans* and *Streptococcus sp. oral clone C3ALM006*, displayed much higher hemolytic activity when they were spotted onto SHI agar plates supplemented with 0.5 mM Bipy, as indicated by the more greenish color zone around the colonies (Fig. 5C).

Effect of Bipy on the salivary microbial community cultivated in chemically defined artificial saliva solution (ASS)

Conditions with limited availability of free iron appear to select for certain oral microbial species, such as *Streptococci cristatus* and *Gemella haemolysans* that were shown to display hemolytic activity (Fig. 5), which could potentially provide additional iron source to sustain

their growth. In addition to their hemolytic activity these species could also have an increased ability to persist in iron-deficient environments which would further enhance their competitiveness. To test this hypothesis, we used ASS medium, a chemically defined medium that was developed in our laboratory for cultivation of oral microbial flora (25). Different concentrations of iron chelator were added to the medium and microbial profiles were monitored by DGGE. Addition of as little as 1 μ M Bipy already started to cause change in community profile resulting in the loss or great reduction of *Campylobacter concisus*, *Veillonella atypica* and *Prevotella* spp. among others (Fig. 6). At a concentration of 50 μ M, Bipy was able to induce a drastic community shift very similar to those observed in SHI medium supplemented with Bipy (Fig. 3), with *Gemella haemolysans*, *Granulicatella adiacens*, and *Streptococci*, including *S. cristatus* becoming the predominant species. More strikingly, at 1 mM of Bipy, a concentration high enough to chelate most of the free ferrous iron (0.1 mM) in the ASS medium, several *Streptococcus* spp. and *Gemella* spp., including *Streptococcus* spp. oral clone C3AKM006 and *G. haemolysans* which could also be selected from Bipy-supplemented SHI medium were still able to grow. Our data suggested that, besides being capable of lysing host red blood cells and retrieving complexed iron, these bacterial species are able to persist under iron-deficient conditions. It is worthwhile to note that higher concentration of Bipy often resulted in reduced biomass (data not shown) both in SHI and ASS medium, confirming that the majority of bacterial species require iron for growth.

DISCUSSION

As one of the most essential trace elements for the growth of virtually all organisms, iron plays an important role in determining the bio-abundance and bio-diversity within a variety of different ecosystems (32–36). However, only a limited number of studies have evaluated the effect of iron availability on the bacterial composition of human-associated microbiota (37). In this study, using *in vitro* human salivary microbial communities, we investigated whether the accessibility of iron plays a role in modulating the microbial populations within the oral microbiota.

We generated culturing conditions with differential free iron availability by adding different concentrations of Bipy, a ferrous iron (Fe^{2+}) chelator to SHI medium containing sheep blood. Our data revealed that titration of free Fe^{2+} from SHI medium resulted in a drastic shift in the bacterial composition of an *in vitro* salivary microbial community, with *Streptococcus* spp, *Gemella* spp. and *Granulicatella* spp. becoming the most dominant bacterial species (Fig. 1). The Bipy-induced shift in microbial composition was observed both in pooled and individual saliva-derived microbial communities (Fig. 1, 3). The similarity in the resultant bacterial profiles among different communities and the seemingly abrupt shift occurring at 0.3 μ M Bipy suggested that, the addition of 0.3 μ M Bipy could reduce the concentration of free iron in the medium below a threshold that might be required for the optimal growth of oral community. Once the free iron level is below the threshold, it could shift the microbial population toward bacterial species that might be more capable of utilizing alternative iron sources and/or more resistant to the iron starvation conditions.

Interestingly, the identified major bacterial species growing under iron-limited condition are phylogenetically related to different branches of *Streptococci* (38–39). *Gemella* spp. was previously classified into the genus *Streptococcus* (40), and reclassified as genus *Gemella* later based on the nucleotide sequence of the 16S rRNA (41). While for *Granulicatella* spp. and *Abiotrophia* spp. (identified from subject 3), they were originally described as “nutritionally variant streptococci (NVS)”, a type of streptococci exhibiting satellitism around colonies of other bacteria (42). These bacterial species share many physiological and biochemical properties with the viridans group streptococci, including the range of

infections that they cause (43). One of the common characteristics among these bacterial species is their α -hemolytic activity (44–46), which was further confirmed by our hemolysis assay results (Fig. 5). The SHI medium used in this study contains 5% sheep blood (24), the abundant iron-containing heme within sheep red blood cells could potentially serve as an iron source for oral bacteria under free iron-limited condition. The α -hemolytic activity would allow bacteria to partially lyse the red blood cells to release iron-complexes, e.g. heme and hemoglobin. Although the mechanisms regarding heme uptake in *Gemella* and *Granulicatella* spp., is currently unknown, many *streptococci* have been shown to employ high-affinity iron uptake systems to obtain heme (20, 47).

Intriguingly, some of the selected *Streptococcus* and *Gemella* strains, including *Streptococcus* spp. oral clone C3AKM006 and *G. haemolysans* isolated from both Bipy-supplemented ASS and SHI medium displayed enhanced α -hemolytic activity under iron-limited condition (Fig. 5C). For many host-associated bacterial species, iron starvation could act as an important environmental cue and induce specific adapted response in bacteria, including the induction of siderophore-mediated iron uptake systems and other potentially pathogenic features (1, 48). Certain *Streptococcus* spp., including *S. pyogenes* displayed increased hemolytic activity due to high hemolysin production when encountering iron-limited condition (49). Our data clearly suggested that, during iron starvation, many oral bacterial species, such as *Gemella haemolysans*, *Granulicatella adiacens*, and certain *Streptococci*, including *S. cristatus* might be able to sustain their growth by retrieving host iron-complexes via hemolytic activity.

Furthermore, when using chemically defined ASS medium without the inclusion of sheep blood, the addition of Bipy resulted in a community with a profile that was very similar microbial to the one obtained with Bipy-supplemented SHI medium (Fig. 6). Even in the presence of Bipy at a concentration that would chelate most of the free Fe^{2+} iron within the medium, several *Streptococci*, and *Gemella* species could still be isolated from the culture. 16s rDNA sequence analysis revealed that their identities matched a subset of *Streptococcus* and *Gemella* strains (including *S. parasanguinis* and *G. haemolysans*) isolated from Bipy-treated SHI medium (data not shown) which had been show to possess hemolytic activity. These results indicated that besides being capable of acquiring host iron-complexes, certain oral strains are also highly resistant to iron starvation conditions. Although iron is required for the growth of the virtually all microbes, different bacteria might have differential iron requirement for sustaining their normal growth and possess different ability to persist under iron deficient condition (1). Furthermore, certain bacterial species, including oral *Lactobacilli* (50) adapt their metabolism towards an absolute manganese requirement instead of absolute iron requirement as a possible defense mechanism against endogenous superoxide during aerobic metabolism (51). However, we did not isolate any *Lactobacilli* strains from salivary communities cultivated using either Bipy-supplemented SHI medium or ASS medium. This could be due to the fact that although SHI medium has been shown to sustain the growth of a variety of oral microbes, it does not seem to favor the growth of *Lactobacilli* (24).

The varying capability of oral bacterial species in exploring iron sources and acquiring iron from their environments, as well as their differential resistance to iron starvation, are likely to make iron, this scarce and growth-limiting trace element, one of the modulating factors in shaping the host associated microbial community. This is corroborated by our observation that, for a mixed community composed of populations obtained from both iron-limiting and iron-containing medium, iron supplementation can restore the original bacterial profile; while the addition of an iron chelator drove the mixed culture towards a community that had been shown to be specifically selected under iron-deficient condition (Fig.4).

The most intriguing finding of this study was that the majority of the oral bacterial species isolated under iron deficient condition have previously been implicated in infectious endocarditis (IE)(52–56). IE is an infection of the lining of the heart chambers and heart valves that is caused by bacteria or other infectious substances. Since the description of the systemic significance of oral infection in late 19th century (57), every major body system has been identified as a potential targeting site for bacterial metastasis of oral origin (58). The involvement of oral bacteria in the pathogenesis of endocarditis had been previously established (52, 58). It has been estimated that 65% of IE clinical cases were due to infection of α -hemolytic streptococci and majority of them were of oral origin. With the improvement of culturing technique and non-culture based detection methods, more and more fastidious bacteria have been identified to be involved in IE, including *Gemella* (59–60), *Granulicatella* (39, 54), as well as *Abiotrophia spp.* (56, 61). Normal oral functional activities like chewing, as well as most dental procedures, such as periodontal cleaning, tooth extraction and endodontic procedures could cause tissue surface trauma and transient bacteremia (62). In individuals with heart malformations or the presence of vascular prostheses, the transient bacteremia could potentially lead to attachment of bacteria and result in IE. It is intriguing to speculate that, for an individual with an oral microbial community containing more IE-related bacterial species, such as α -hemolytic bacteria as a result of particular oral or systemic conditions, there could be a potentially higher risk of endocarditis. Further investigation is needed to determine whether there is a possible association between the saliva iron content and individual's risk of infectious endocarditis.

The human oral cavity represents one of the most complex host-associated microbial ecosystems ever identified. Like gut-associated microbial flora, the microbial composition within the oral habitat could be a complex polygenic trait shaped and modulated by multiple host and environmental factors. A healthy and balanced oral commensal flora with stable microbial composition could play an important role in maintaining the community stability and preventing foreign/pathogenic colonization; while unbalanced population structure due to local or systemic conditions could lead to the deterioration of oral ecological conditions and induce adverse effect on the host. Our data suggested that, as one of the most essential trace elements, the accessibility of free iron could play an important role in modulating the population structure of the commensal oral microbiaota and have potential clinical relevance in inducing certain disease conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PCR-DGGE analysis of microbial profiles of pooled saliva cultivated in SHI medium in the presence of the iron chelator 2,2' bipyridyl (Bipy)

Each lane represents pooled saliva cultured in SHI medium containing the specified amounts of 2,2'-bipyridyl. The arrows indicate the dominant bands whose corresponding bacterial ID was determined at species level when their sequenced 16S fragments have more than 98% identity to those deposited in the database. For those that have multiple hits with same identity, both bacterial species are listed and separated with "/"; while "spp." was used when their sequences have less than 98% identity to that deposited in the database. Two biological replicates were performed for each experiment and a representative gel image is shown.

Figure 2. PCR-DGGE analysis of microbial profiles of pooled saliva cultivated in SHI medium supplemented with or without Bipy (0.3 mM) and/or Fe²⁺ (0.1 mM)

Addition of Bipy or Fe²⁺ is indicated by (+), while (–) represents samples without exogenously added Bipy or Fe²⁺. Two biological replicates were performed and a representative gel image is shown.

Figure 3. Effect of the iron chelator Bipy on the microbial profiles of salivary communities from different subjects

Individual saliva samples were cultivated in SHI medium in the presence and absence of exogenously added Bipy (0.3 mM). Overnight cultures were subjected to PCR-DGGE analysis. Addition of Bipy is indicated by (+), while (–) represents samples without exogenously added Bipy. The arrows indicated the dominant bands whose corresponding bacterial ID was determined. Two biological replicates were performed and a representative gel image is shown.

Figure 4. The modulating effect of iron availability on the salivary microbial communities
 Pooled saliva samples were cultivated in the absence and presence of Bipy (0.3 mM) overnight. The resulting cultures, culture A and culture B, respectively, were mixed at a 1:1 ratio, and further cultivated for 16 hrs with the addition of either Bipy (0.3 mM) or Fe²⁺ (0.1 mM). Samples were taken and subjected to PCR-DGGE analysis. Two biological replicates were performed and a representative gel image is shown.

Fig. 5. Hemolytic activity of salivary communities and individual isolates under different culture conditions

A) Hemolytic activity of cultivated salivary communities: Salivary cultures in SHI medium supplemented with Bipy (0.3 mM) or Fe²⁺ (0.1 mM) were diluted and spotted onto SHI medium agar plates with 0.5% sheep blood. Plates were incubated at 37°C under anaerobic condition for 24 hrs. Addition of Bipy or Fe²⁺ is indicated by (+), while (-) represents samples without exogenously added Bipy or Fe²⁺. B) Hemolytic activity of salivary isolates. Salivary isolates from cultures supplemented with and without Bipy were cultivated and spotted onto solid SHI medium with 0.5% sheep blood. Plates were incubated at 37°C under anaerobic condition for 24 hrs before hemolytic activity was recorded. C) Hemolytic activity of salivary isolates on SHI agar plates in the presence and absence of 0.5 mM Bipy. Two biological replicates were performed for each setup and representative images are shown.

Figure 6. PCR-DGGE analysis of microbial profiles of pooled saliva cultivated in ASS medium with increasing concentrations of Bipy

Each lane represents pooled saliva cultured in ASS medium containing the specified amounts of 2,2'-bipyridyl. The arrows indicate the dominant bands whose corresponding bacterial ID was determined and shown. Two biological replicates were performed and a representative gel image is shown.