〔REVIEW〕

Prospects and Significance of Oral *Veillonella*

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Abstract

Oral biofilm causes many oral infections, such as dental caries and periodontal disease. Oral *Veillonella* has been suggested to have important roles in the early stages of oral biofilm formation. Therefore, it is necessary to reveal the functional mechanisms of oral *Veillonella* in oral biofilm formation to prevent many oral infections.

In this article, I summarize the characteristics of genus *Veillonella*, especially the oral *Veillonella* species, based on classification and phenotypical identification methods. This article also reviews recent findings, such as specific PCR primers to identify oral *Veillonella* at the genus and species level, the distribution and frequency of oral *Veillonella* on the tongue, in the saliva, and in subgingival biofilm, and the relationship, involving autoinducers, between oral *Veillonella* species and oral *Streptococcus* species reported as initial colonizers in biofilm formation produced by *Veillonella* species. Furthermore, this review introduces interesting results obtained by 16S rRNA metagenomic analysis of salivary samples, and the possibility of the occurrence of many novel oral *Veillonella* species demonstrated in phylogenetic studies based on *rpoB* and *dnaK* gene sequence analysis.

Although these new findings reviewed in this article are very important, they are not sufficient to understand the role of oral *Veillonella* in oral biofilm formation. New knowledge on oral *Veillonella* needs to be gathered which might help in the preventing oral biofilm formation and cure of oral infections.

Introduction

The human oral cavity contains more than 19,000 microbial phylotypes (Keijser et al., 2008). Bacterial communities in oral cavities are the second most complex bacterial flora in the human body (Human Microbiome Project Consortium, 2012). These bacterial members of the communities form biofilms in the human oral cavities. Dental plaque is a well–recognized example of a natural biofilm as oral biofilm. It is generally known that bacteria, which form biofilms, are not easily eliminated by the immune response and are resistant to antimicrobial agents (Bjarnsholt et al., 2013).

Oral biofilms are composed of multiple species of bacteria, whose development is initiated by the adherence of pioneer species to salivary proteins and glycoproteins adsorbed on tooth enamel. The biofilm is not formed by the random simultaneous colonization of these species, but rather by selective, reproducible, and sequential colonization (Bjarnsholt et al., 2013 ; Nyvad & Kilian, 1987). In addition, oral biofilm is the source of bacteria that causes oral infections, such as dental caries and periodontal disease, and sometimes leads to systemic diseases or makes them worse (Maddi & Scannapieco, 2013).

Periasamy & Kolenbrander (2010) reported that *Veillonella* species has a central role in oral biofilm formation as an early colonizer together with *Streptococcus* species reported as initial colonizer to be involved in establishing multispecies oral biofilm communities. Therefore, it is important to understand the role of oral *Veillonella* species for oral biofilm formation for treatment and prevention of oral infectious diseases. However, both the pathogenic and congenital roles of oral *Veillonella* species in oral biofilm formation has not been sufficiently revealed, nor has the distribution and frequency of oral *Veillonella* species been clari-

fied at the species level.

This article reviews recent studies related to oral *Veillonella* species, including our studies, and reflects upon the current state of oral *Veillonella* research.

1. Genus *Veillonella*

The genus *Veillonella*, named after Adrien Veillon, contains multiple species of gram−negative, obligately anaerobic, non−motile, non−spore−forming, small cocci belonging to the family Veillonellaceae, order Clostridiales, class Clostridia, and phylum Firmicutes (Jean−Philippe, 2009).

Thirteen species are established within the genus including *Veillonella atypica*, *Veillonella caviae*, *Veillonella criceti*, *Veillonella denticariosi*, *Veillonella dispar*, *Veillonella magna*, *Veillonella montpellierensis*, *Veillonella parvula*, *Veillonella ratti*, *Veillonella rodentium*, *Veillonella rogosae*, *Veillonella seminalis*, and *Veillonella tobetsuensis* (Arif et al., 2008 ; Aujoulat et al., 2014 ; Byun et al., 2007 ; Jumas−Bilak et al., 2004 ; Kraatz & Taras, 2008 ; Mashima et al., 2013 ; Mays et al., 1982 ; Rogosa, 1965).

Veillonella species, except *V. criceti*, *V. ratti*, and *V. seminalis*, do not ferment carbohydrates or amino acids, and they lean the fermentation of pyruvate, lactate, malate, fumarate, and/or oxaloacetate as a source of carbon and energy. Major metabolic end products of members of the genus *Veillonella* are acetic acid and propionic acid from trypticase−glucose−yeast extract. Their DNA G+C content is 36 −43% mole, and the type species is *V. parvula* (Jean− Philippe, 2009).

Veillonella species are present in the oral cavity, intestinal tracts, and genitourinary and respiratory systems of humans and animals as commensal organisms. Some *Veillonella* species have been reported as rare causative organisms of meningitis, endocarditis, bacteremia, discitis, vertebral osteomyelitis, and prosthetic joint infection (Isner−Horobeti, 2012 ; Liu, 1998 ; Marriott et al., 2007). In addition, *Veillonella* species are known as opportunistic pathogens with the ability to take advantage of a host's weakened immune function to cause infections (Aas et al., 2007 ; Fisher and Denison, 1996).

Generically, *Veillonella* species are resistant to tetracycline and sensitive to penicillin and ampicillin. However, some *Veillonella* strains have recently been found to be resistant to these two antibiotics (Readyy et al., 2012). Furthermore, it is reported that one of the pathogenicity of *Veillonella* species is related to its lipopolysaccharide (LPS). Fructose is incorporated into LPS of *Veillonella*, and may be of major significance in the production of endotoxic LPS (Tortorello & Delwiche, 1983).

2. Oral *Veillonella*
1) Classification and Biology

Of the 13 established species, *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* have been isolated from human oral cavities, and these oral *Veillonella* species are mainly located in the dental biofilms, tongue biofilms, buccal mucosa, saliva, periodontal pockets, and caries lesion of dentin (Dongyou, 2011).

Oral *Veillonella* species have diverse roles in polymicrobial disease. These species utilize lactate produced by carbohydrate−fermenting *Lactobacillus* species and *Streptococcus* species, and the lactate is converted into the weaker acid and propionic acid. Consequently, *Veillonella* species reduce the concentration of lactate, and ameliorate the process of dental caries because the weaker acid has less capability to decalcify teeth (Dongyou, 2011).

Oral *Veillonella* species produce volatile sulfur compounds, which causes oral malodors in the polymicrobial community of the oral biofilm. In addition, hydrogen sulfide produced by oral *Veillonella* from L−cysteine in the presence of human serum is toxic to host cells and contributes to its disease−inducing potential (Washio et al., 2005).

Porphyromonas gingivalis, *Prevotella intermedia*, and *Treponema denticola* are known as pathogenic microorganisms for periodontal disease (Dongyou 2011 ; Socransky & Haffajee, 1992). Oral *Veillonella* species produce menaquinones, and supply the essential nutritional requirement for growth of *Porphyromonas* and *Prevotella* species. In addition, oral *Veillonella* species provides different peptidases, which complements the activity and promotes the growth of *T. denticola* (Dongyou, 2011).

2) Phenotypic Identification

Veillonella species are isolated on a selective medium, *Veillonella* agar, which is composed of Bacto tryptone, Bacto yeast extract, sodium thioglycolate, basic fuchsin, sodium lactate, agar, and vancomycin (Rogosa et al., 1958). The inoculated plate is incubated under obligately anaerobic conditions, such as 80% N_2, 10% CO_2, and 10% H_2, at 37°C for 5 days. Colonies on *Veillonella* agar are presumptively

identified as *Veillonella* strains on the basis of their ability to grow on the selective medium. Typical colonies are opaque or grayish−white, 2–4 mm in diameter, regular, and slightly domed in shape with an entire edge. In addition, they lack hemolytic activity in blood agar (Jean−Philippe et al., 2009).

Conventional biochemical analysis was performed on cells grown on *Veillonella* agar using the rapid ID 32A identification kit and API ZYM (bioMe´rieux) (Jean−Philippe et al., 2009). For further examination of the *Veillonella* strain, an electron microscope was used. The ultrathin sections revealed cell structural components, such as outer membrane, a thin peptidoglycan layer, and cytoplasmic membrane, which are characteristic of a gram−negative bacterial cell wall and consistent with the genus *Veillonella* (Jean−Philippe et al., 2009).

In some cases, cellular fatty acids of *Veillonella* were analyzed after methyl esterification. Major cellular fatty acids are $C_{13:0}$ and $C_{17:1\omega8}$, and minor or trace fatty acids are $C_{11:0}$, $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1\omega9c}$, $C_{16:0}$, $C_{17:0}$, $C_{18:1\omega9c}$, and $C_{18:0}$ (Byun et al., 2007 ; Jean−Philippe et al., 2009 ; Jumas−Bilak et al., 2004 ; Mashima et al, 2013).

3) Genotypic Identification

Phenotypic methods are effective to identify members of *Veillonella* species at genus level, but do not allow differentiation among members of the genus *Veillonella* at species level. Therefore, molecular techniques, such as DNA−DNA hybridization, PCR with specific primers, PCR−random fragment length polymorphism analysis, and sequencing based on the 16S rRNA gene, have been developed for identification of *Veillonella* species (Marchandin et al., 2003 ; Rolph, 2001 ; Sato et al., 1997 ; Siqueira, 2001). Although it is known that 16S rRNA gene is useful for identification of some bacterial species, 16S rRNA gene comparison of *Veillonella* has not been reliable for identification at the species level. This is because *Veillonella* species have highly conserved sequences in 16S rRNA gene. In addition, there is a high level of intrachromosomal heterogeneity in some oral *Veillonella* species (Byun et al., 2007 ; Jumas−Bilak et al., 2004).

Therefore, some housekeeping genes, such as *rpoB*, *dnaK*, *gyrB*, and *gltA*, have been used for more veracious identification of *Veillonella* species (Arif et al., 2008 ; Aujoulat et al., 2014 ; Byun et al., 2007 ; Dongyou, 2011 ;

Kraatz & Taras, 2008 ; Mashima et al., 2013 ; Michon et al., 2010). The *dnaK* gene, coding heat−shock protein 70 kD, has higher interspecies variability than 16S rRNA gene. Comparison of *dnaK* gene sequences aids classification of the known members of genus *Veillonella*. Furthermore, sequencing of the *rpoB* gene, which encodes an enzyme that synthesizes RNA polymerase subunit B, appears to be more effective than *gyrB* and *gltA* gene sequencing for identification of *Veillonella* at species level (Aujoulat et al., 2014 ; Dongyou L. 2011 ; Jean−philippe et al., 2009).

4) *Veillonella* Genus−specific PCR Primers

Classification of *Veillonella* species at the genus level is not difficult based on the phenotypic characteristics of bacteria, such as colony appearance, arrangement and size of the bacterial cells, and gram−negative cocci, cultured on the selective medium *Veillonella* agar under the strictly anaerobic conditions. On the other hand, Arif et al. (2008) established a genus−specific PCR primer set based on the sequence of *rpoB* gene for members of genus *Veillonella* : Veill−rpoBF (5'−GTAACAAAGGTGTCGTTTCTCG−3') and Veill−rpoBR (5'−GCACCRTCAAATACAGGTGTAGC−3') (Arif et al., 2008). Thereafter, Mashima et al. (2011 ; 2013 ; 2015) and Theodorea et al. (2017) demonstrated the availability and accuracy of the genus−specific PCR primer set (Mashima et al., 2011, Mashima et al., 2013 ; Mashima et al., 2015 ; Theodorea et al., 2017).

5) Distribution and Frequency with the Species−specific PCR Primers

Beighton et al. (2008) reported the predominant cultivable *Veillonella* species in tongue biofilm from healthy adults using *rpoB* gene sequence analysis. In their report, *V. atypica*, *V. dispar*, and *V. rogosae* were the predominant species, and *V. denticariosi* was not identified at all. In addition, Arif et al. (2008) investigated the diversity of *Veillonella* species in caries sites in children using 16S rRNA, *dnaK*, and *rpoB* gene sequence analysis. The proportion of *Veillonella* species was not significantly different between the caries and caries−free groups, but *V. denticariosi* was only detected in the caries group and *V. rogosae* was only detected in the caries−free group (Arif et al., 2008). Although this study was beneficial to help discriminate among oral *Veillonella*, more affordable, time−saving, and easy handling methods are needed to identify oral *Veillonella* species.

Igarashi et al. (2009) showed that five oral *Veillonella* species, *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, and *V. rogosae*, could be readily identified via two−step PCR using five forward primers from a highly variable region of the *rpoB* gene and one common reverse primer shown in Table 1. Mashima et al. (2011) examined the distribution and frequency of oral *Veillonella* species in the tongue biofilm of healthy 20 adults. They isolated 416 strains from 27 subjects, and 404 isolates were identified using the species−specific primer sets designed by Igarashi et al. (2009) as either *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, or *V. rogosae*. They demonstrated that *V. atypica*, *V. dispar*, and *V. rogosae* were detected as the predominant oral species throughout all samples, and *V. parvula* and *V. denticariosi* were isolated from limited subjects. Furthermore, they reported that the tongue biofilms of these subjects were divided into two groups based on the distribution and frequency of oral *Veillonella* species, which meant that *V. rogosae* was the predominant species in one group ; the other group consisted mainly of *V. atypica* and *V. dispar* (Mashima et al., 2011). This result partially supported the report by Beighton et al. (2008).

Recently, *Veillonella tobetsuensis* isolated from human tongue biofilm was established as a novel *Veillonella* species (Mashima et al., 2013). Thereafter, the distribution and frequency of *V. tobetsuensis* in human tongue biofilms were determined using a *V. tobetsuensis*−specific PCR primer pair designed based on the sequence of the *dnaK* gene (position 424−1048). The forward primer was VTF (5'−CTCTCAAC GTCAAGCAACAAAAGATGC−3'). The reverse primer was VTR (5'−GATAAGGTAGTTCATGATGCGTTGG−3'). In that study, it was demonstrated that *V. tobetsuensis* was detected in 5 of 27 subjects (19%), and its prevalence ranged from 7.6% to 20.0% (Mashima et al., 2013).

The distribution and frequency of six oral *Veillonella* species *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* in the subgingival biofilm from periodontal pockets were also determined by PCR with the species−specific primers (Igarashi et al., 2009 ; Mashima et al., 2013). In the study, it was demonstrated that the frequency of *V. parvula* was significantly higher in the periodontal pockets than that in the gingival sulcus at the species level, which suggested that *V. parvula* is associated with a state of chronic periodontitis (Mashima et al., 2015).

Recently, the relationship between the frequency of six oral *Veillonella* species and different oral hygiene states was determined in Thai children divided into three groups, Good, Moderate, and Poor oral hygiene group, based on the Sim-
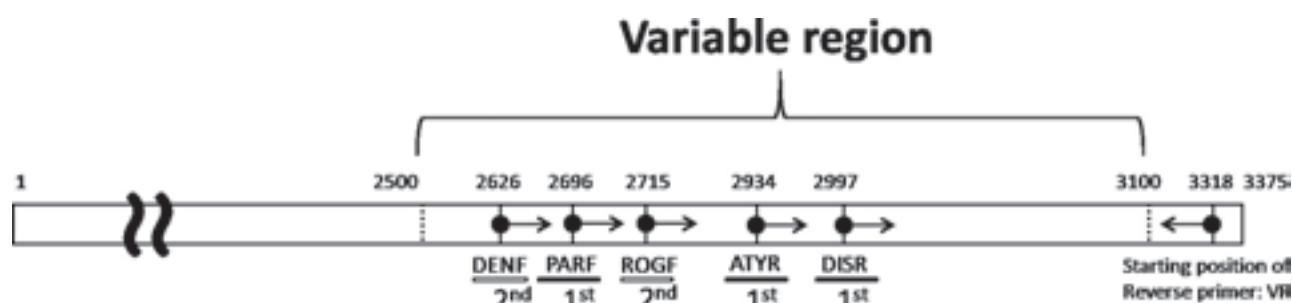
Primer name	Target	Sequence
ATYF	*V. atypica*	5'-TCTCTTTGGGAAGAATTAGAACGC-3'
DENF	*V. denticariosi*	5'-GAAAGAAGCGCGCACCGACAGT-3'
DISF	*V. dispar*	5'-AACGCGTTGAAATTCGTCATGAAC-3'
PARF	*V. parvula*	5'-GAAGCATTGGAAGCGAAAGTTTCG-3'
ROGF	*V. rogosae*	5'-ATTGCAGAAGATGTAACATGAAGC-3'
VR	All 5 species	5'-GTGTAACAAGGGAGTACGGACC-3'

Table 1 : Primer sequences for two−step species−specific PCR (Igarashi et al., 2009) designed using the variable region of the *rpoB* gene.
ATYF, DISF, PARF, and VR are used for the 1st step PCR, and DENF, ROGF, and VR are used for the 2nd step PCR.

plified Oral Hygiene Index (OHI–S) (Mashima et al., 2016 ; Theodorea et al., 2017). Tongue biofilms from 89 children and saliva samples from 107 children were collected in these studies, and the distribution and frequency of six oral *Veillonella* species were examined by PCR identification. Mashima et al. (2016) established a novel one–step PCR method with one forward primer and six reverse primers designed using the sequence of the conserved region or variable regions in the *rpoB* gene of oral *Veillonella* species shown in Table 2.

In case of tongue biofilms, oral *Veillonella* isolates were detected predominantly in subjects who had Poor oral hygiene compared to those with Good or Moderate oral hygiene. At the species level, although *V. atypica*, *V. dispar*, *V. rogosae*, and *V. tobetsuensis* were detected in only 10 of the 89 subjects, *V. rogosae* was the predominant species in all groups. *V. dispar* was detected mainly from subjects who had Good or Moderate oral hygiene group. However, *V. parvula* and *V. denticacariosi* were not isolated from any subjects. As conclusion, they suggested a possibility that these *Veillonella* species might be an index for a person's oral hygiene state (Mashima et al., 2016).

In case of saliva samples, *Veillonella* species were detected in all subjects from three groups ; the total number of isolates was 1609 from 107 subjects. Six oral *Veillonella* species were identified in 1442 of 1609 isolates, and 167 isolates were unclassified. The prevalence of *V. rogosae* was significantly lower in the Poor oral hygiene group than that in the Good oral hygiene group. On the other hand, *V. tobetsuensis* was not detected in the Good oral hygiene group. Although *V. rogosae* was the predominant species in all groups, *V. parvula*, *V. tobetsuensis*, and the unclassified *Veillonella* isolates were significantly more prevalent in the Poor oral hygiene group, which suggested that the detection rate of *V. parvula* and *V. tobetsuensis* in saliva indicates the oral hygiene state in children (Theodorea et al., 2017).

6) Biofilm Formation and Autoinducer

The human oral biofilm is a multispecies community formed by more than 700 bacterial species, including 19,000 microbial phylotypes (Hajishengallis & Lamont, 2012 ; Huang & Gregory, 2011). In the process of dental biofilm formation, oral *Streptococcus* species attach to the tooth surface covered by an acquired pellicle with specific interactions as pioneer colonizers. The growth of the pioneer colonizers establishes an environment conducible for the colonization by the bridging bacterial species, some of which are *Veillonella* species as early colonizers (Diaz et al., 2006 ;
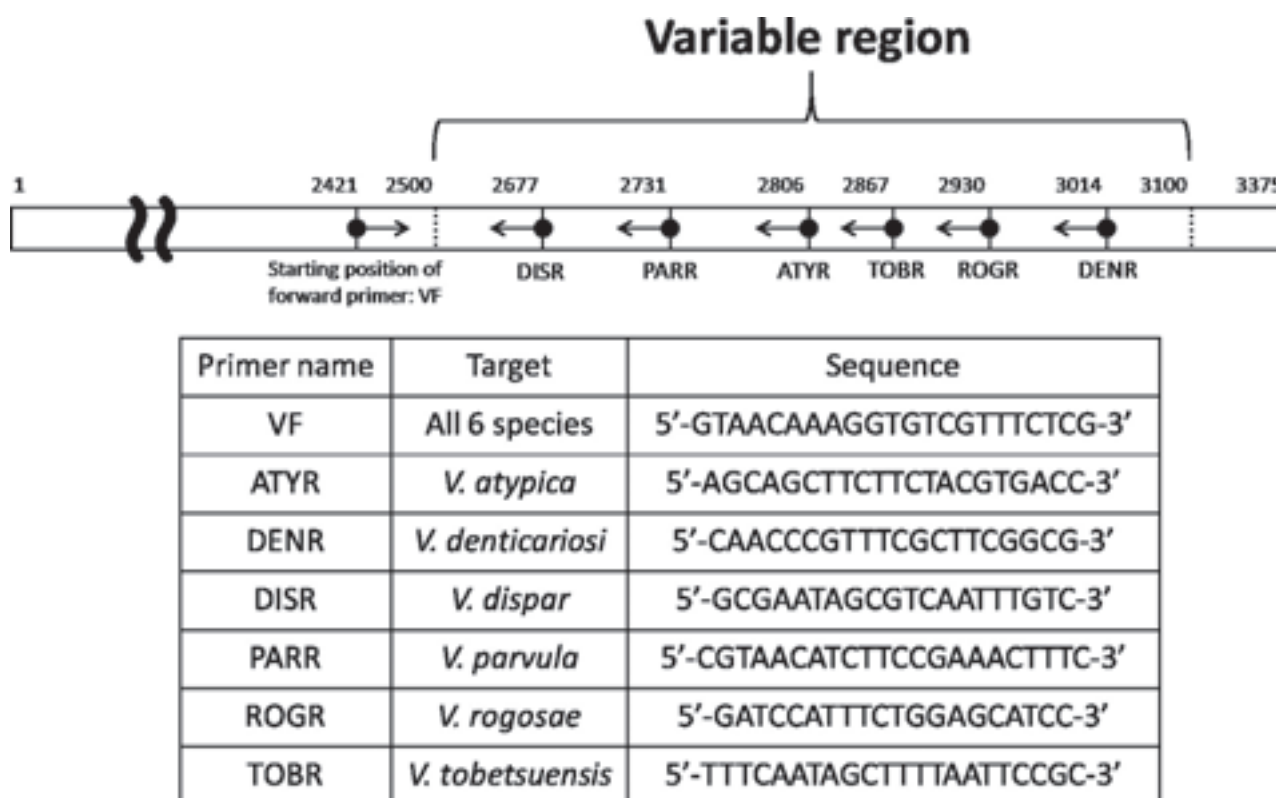

Primer name	Target	Sequence
VF	All 6 species	5'-GTAACAAAGGTGTCGTTTCTCG-3'
ATYR	*V. atypica*	5'-AGCAGCTTCTTCTACGTGACC-3'
DENR	*V. denticariosi*	5'-CAACCCGTTTCGCTTCGGCG-3'
DISR	*V. dispar*	5'-GCGAATAGCGTCAATTTGTC-3'
PARR	*V. parvula*	5'-CGTAACATCTTCCGAAACTTTC-3'
ROGR	*V. rogosae*	5'-GATCCATTTCTGGAGCATCC-3'
TOBR	*V. tobetsuensis*	5'-TTTCAATAGCTTTTAATTCCGC-3'

Table 2 : Primer sequences for the one–step PCR method (Mashima et al., 2016) designed using the variable region of the *rpoB* gene.

Kolenbrander et al., 2006 ; Kolenbrander et al., 2010). Subsequently, the bridging bacteria grow and create a local environment to facilitate attachment and growth of middle and late colonizers, such as periodontopathogenic bacterial species, for maturation of the oral biofilm (Zhou et al., 2016).

For *in vitro* biofilm experiments, microtiter plate−based biofilm assays have been widely used for long time (Christensen et al., 1985). A flow cell system has also been used frequently for *in vitro* biofilm experiments (Foster & Kolenbrander, 2004). Although these methods certainly have merit, many limitations, such as technical sensitivity or high cost performance, of these methods prohibit routine use. To overcome these limitations, Mashima & Nakazawa (2012) established a novel method for the formation of a biofilm in an *in vitro* experiment using the "wire method" (Fig. 1). In the wire method, a cobalt−chrome alloyed wire, 0.9 mm in diameter and 15 cm in length, was connected to a rubber plug on a test tube. The wire was inserted into a bacterial suspension in the test tube as a scaffold for biofilm formation. After incubation, the wire with biofilm was transferred to new tube, and the biofilm was removed from the wire using a short silicone tube (TYGON 3350) which was set on the wire. Afterwards, DNA was extracted from the bacterial cells of the harvested biofilm to quantify the number of bacteria in the biofilm. Currently, a wire at half the size (7.5 cm) with a silicone plug is used in our laboratory for easier handling, and similar results have been obtained in experiments as those obtained in experiments with the original size wire (15 cm) (Fig. 1).

The *Veillonella* species, one of the most predominant bacteria in the human oral microbiome, produces nutrients for growth of late colonizers (Aas et al., 2005 ; Aas et al., 2008 ; Becker et al., 2002). In addition, *Veillonella* species have two important characteristics as a "bridging species" for maturation of the oral biofilm community : 1) *Veillonella* species use lactate produced by *Streptococcus* and *Lactobacillus* species (Rogosa, 1964) and 2) *Veillonella* species can adhere to initial, middle, and late colonizers (Hughes et al., 1988 ; Chalmers et al., 2008 ; Periasamy & Kolenbrander, 2010 ; Zhou et al., 2015). Therefore, *Veillonella* species have an important role in the development of oral biofilm and the ecology of the human oral cavity.

To reveal the role of oral *Veillonella* species as early colonizers versus *Streptococcus* species as initial colonizers, biofilms formed by the co−culture of four oral *Streptococcus* species, *S. gordonii*, *S. mutans*, *S. salivarius*, or *S. sanguinis* in combination with six oral *Veillonella* species, *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, or *V. tobetsuensis* for a total of 24 combinations were studied by the novel wire method (Mashima and Nakazawa, 2014). Type strains, except *S. mutans* ingbritt, were used, and it was demonstrated that the amount of biofilm formed by *S. mutans*, *S. gordonii*, or *S. salivarius* in the presence of six *Veillonella* species was greater than that formed without *Veillonella* species. On the other hand, in the case of biofilms formed by *S. sanguinis*, the presence of *Veillonella* species reduced the amount of biofilm except by *V. parvula* and *V. dispar*. Time−dependent changes in the amount of biofilm and the number of planktonic cells were grouped into four patterns from these 24 combinations (Fig. 2). Only
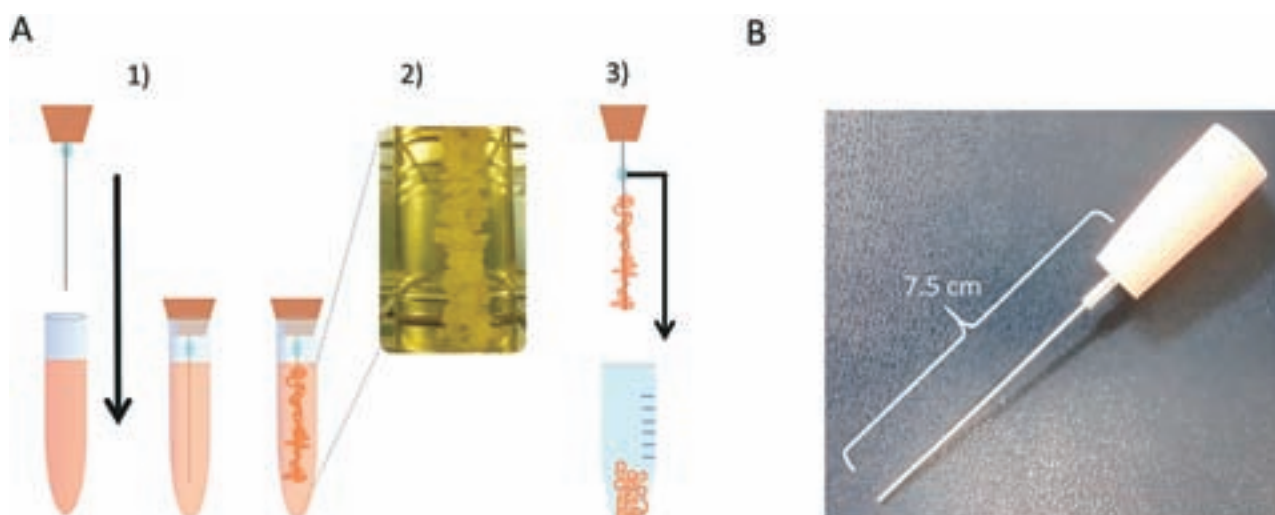

Fig. 1 : A) Scheme for the wire method : 1) the wire is inserted into a bacterial suspension in a test tube, 2) the biofilm forms on the surface of the wire during incubation, and 3) the biofilm is removed from the wire. B) The current wire size used in our laboratory.

that of *S. gordonii* with *V. tobetsuensis* had a unique pattern. In addition, the amount of the biofilm formed by *S. gordonii* alone decreased over time, whereas that formed by *S. gordonii* with *V. tobetsuensis* increased significantly over time. Furthermore, the proportion of *V. tobetsuensis* cells in this biofilm increased over time. These results indicated that *V. tobetsuensi*s promotes the development of biofilms, which were initially formed by *S. gordonii* (Mashima & Nakazawa, 2014).

Taken together, two hypotheses were suggested : (1) coaggregation between *S. gordonii* and *V. tobetsuensis* leads to the formation of a biofilm and (2) *V. tobetsuensis* produces certain signals, such as autoinducers (AIs), that promote biofilm formation by *S. gordonii*.

V. tobetsuensis and *S. gordonii* showed no coaggregation (Mashima and Nakazawa, 2017), which indicates that coaggregation between *V. tobetsuensis* and *S. gordonii* was not a factor to promote their biofilm development. Zhou et al., (2015) investigated the sialic acid binding protein Hsa in *S. gordonii* DL1, which mediates intergeneric coaggregation with *Veillonella* spp. They showed that Hsa was essential for coaggregation between *S. gordonii* DL1 and *Veillonella* spp., but the detailed binding mechanism has not been clarified (Zhou et al., 2015). Hughes et al. (1992) suggested that a certain adhesin of *V. atypica* PK1910 mediated coaggregation with *Streptococcus* spp. (Hughes et al., 1992). These results suggest that coaggregation between *Veillonella* spp. and *Streptococcus* spp. depends on the kind of species or strain. Furthermore, the mechanism of coaggregation varies

and more investigation is needed.

It is well known that AIs, such as AI−1 and AI−2, have important roles in biofilm formation with quorum sensing (QS). In particular, AI−2 is produced by both gram−negative and gram−positive microorganisms, and is a universal QS signal to mediate interactions among bacterial species. In the case of oral bacteria, Rickard et al. (2006) demonstrated that *Streptococcus oralis* and *Actinomyces naeslundii* formed a small amount of biofilm when grown alone, but developed into dense biofilms when grown together via production of AI−2 from *Streptococcus oralis* (Rickard et al., 2006). Yoshida et al. (2005) suggested that AI−2 from *Streptococcus mutans* regulated sucrose−dependent biofilm formation through the expression of a glucosyltransferase gene (Yoshida et al., 2005). Furthermore, it was reported that amount of biofilm formed by *Porphyromonas gingivalis*, which causes periodontal disease, was controlled by AI−2 from *Streptococcus gordonii* (McNab et al., 2003). In addition, an AI−2−like molecule was detected by a *Vibrio harveyi* BB170 reporter assay in large amounts in the supernatant of *V. tobetsuensis* in the mid−exponential growth phase. The AI−2−like molecule was partially purified from *V. tobetsuensis* supernatant, the effect of the AI−2−like molecule from *V. tobetsuensis* on *S. gordonii* biofilm was examined using the wire method, and significantly inhibited *S. gordonii* biofilm development without bactericidal effects (Mashima & Nakazawa, 2017). Jang et al. (2013) reported similar results using *Fusobacterium nucleatum*. They showed that AI−2 from *F. nucleatum* reduced *S. oralis*
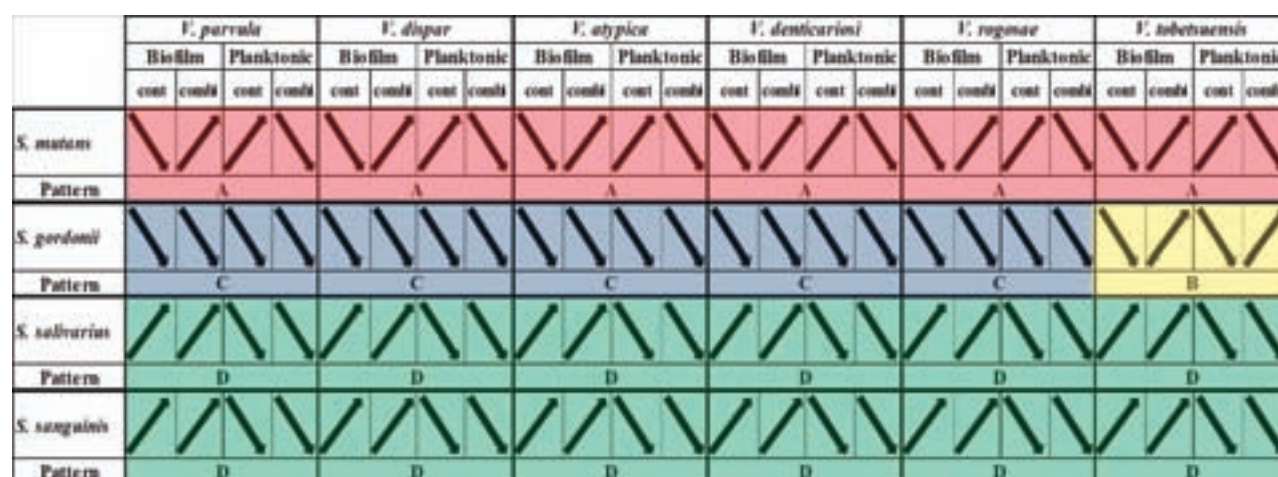

Fig. 2 : Four patterns A, B, C, and D based on time−dependent changes in biofilm formation and the number of planktonic cells among 24 combinations of four *Streptococcus* species and six *Veillonella* species.
Arrows indicate increase or decrease of biofilm development or planktonic cell growth. Cont and Combi indicate Control and Combination, respectively.

biofilm development, as well as the attachment of *F. nuclea-tum* to preformed *S. oralis* biofilms (Jang et al., 2013). Our findings support the additional role of AI−2 as an inhibitor of oral biofilm formation.

Recently, cyclic dipeptides (also known as 2, 5−dike-topiperazines), which are low molecular−weight compounds that have a multitude of bioactive properties produced by many gram−negative bacteria, have also been recognized as AIs (Gu et al., 2013). For example, Marchesan et al. (Marchesan et al., 2015) reported that two cyclic dipeptides, cyclo (−L−Leu−L−Pro) and cyclo (−L−Phe−L−Pro), inhibited the growth of commensal bacteria in the biofilm formed by *Synergistetes* in a biofilm−overgrowth model. In our latest study, a cyclic dipeptide was also detected in the culture su-pernatants from *V. tobetsuensis* at the late−exponential growth phase. It was identified as cyclo (−L−Leu−L−Pro) by GC−MS. In addition, it was demonstrated that although the cyclo (−L−Leu−L−Pro) had no effect on planktonic bacterial cells of *S. gordonii*, the development of biofilm formed by *S. gordonii* was inhibited by the cyclo (−L−Leu−L−Pro) (Mashima et al., unpublished data).

Our previous studies indicated that *V. tobetsuensis* pro-motes the development of biofilms initially formed by *S. gordonii* without coaggregation (Mashima & Nakazawa,

2014 ; Mashima & Nakazawa, 2017). However, the AI−2−like molecule and the cyclo(−L−Leu−L−Pro) produced by *V. tobetsuensis* inhibited biofilm development of *S. gordonii*. Therefore, other extra cellular molecules from *V. tobetsuen-sis* to promote *S. gordonii* biofilm development need to be investigated in the future.

7) Metagenome Analysis

Recently, high−throughput methods have been developed for epidemiologic investigation of microbiome profiles re-lated to disease risk (Jo et al., 2016 ; Gao et al., 2017). In the latest study, we characterized the salivary microbiome in Thai children stratified by oral hygiene state. The samples were collected from 90 Thai children divided into three groups – Good, Moderate, and Poor – based on the results of oral examination using OHI−S. The bacterial community structure of saliva from each individual was analyzed using Illumina MiSeq high−throughput sequencing based on the V 3 and V4 region of bacterial 16S rRNA (Mashima et al., 2017). Twenty taxonomic groups including genera, families, and class for *Streptococcus*, *Veillonella*, *Gemellaceae*, *Prevotella, Rothia*, *Porphyromonas*, *Granulicatella*, *Actino-myces*, TM−7−3, *Leptotrichia*, *Haemophilus*, *Selenomonas*, *Neisseria*, *Megasphaera*, *Capnocytophaga*, *Oribacterium*,
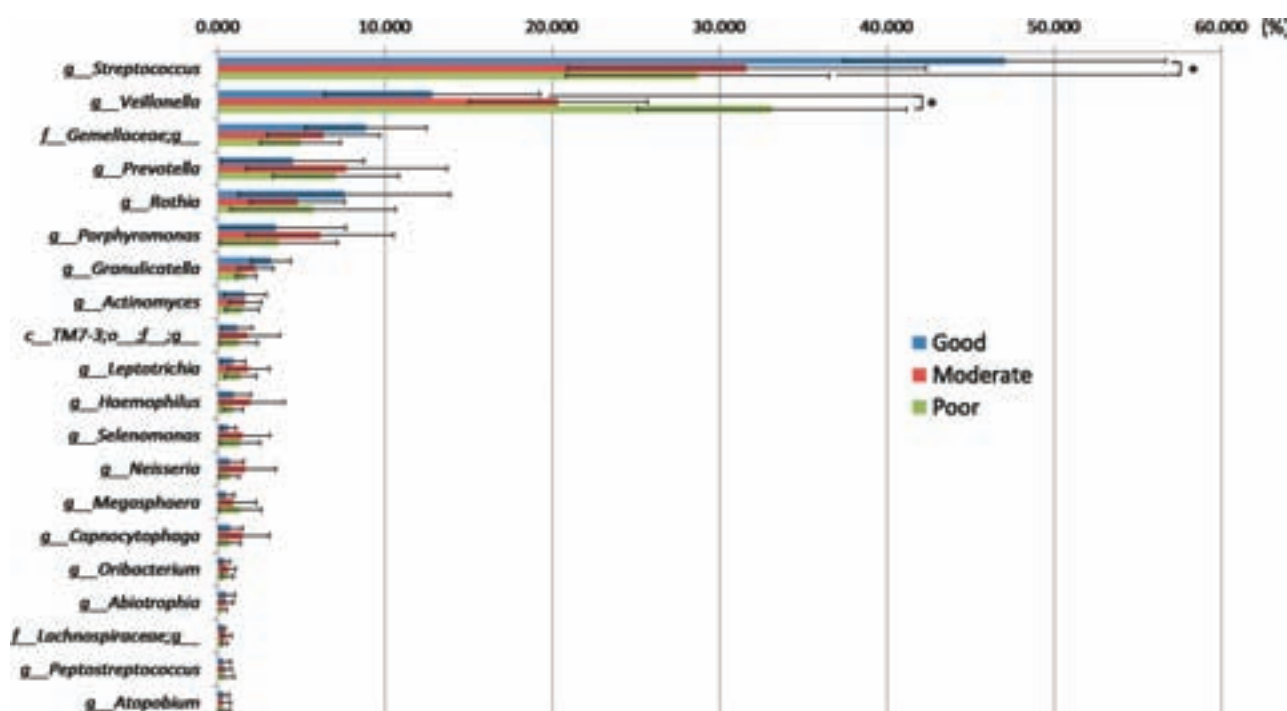

Fig. 3 : Relative abundance of the top 20 bacterial taxa (OTUs) among three oral hygiene groups.
c, *f*, and *g* indicated class, family, and genus, respectively. Some OTUs were not assigned at the genus level based on the database. Sig-nificant differences were evaluated using the Kruskal−Wallis H−test post hoc Mann−Whitney U−test with Bonferroni correction ($P <$ 0.05). Error bars indicate 95% confidence intervals.

Abiotrophia, *Lachnospiraceae*, *Peptostreptococcus*, and *Atopobium* were found in all subjects on the basis of 38,521 OTUs (Operating Taxonomic Units) with 97% similarity. Of these bacterial groups, genus *Streptococcus* had the highest relative abundance (47.5%) in the Good oral hygiene group, and genus *Veillonella* had the highest relative abundance (33.5%) in the Poor oral hygiene group. In other words, the proportion of *Streptococcus* decreased and *Veillonella* increased with poor oral hygiene (Fig. 3). Finally, the study demonstrated an important association between an increase of *Veillonella* and poor oral hygiene in children.

Previously, Takeshita et al. (2009) reported that salivary microbiomes with larger proportions of *Prevotella* and *Veillonella* were associated with periodontitis. Similarly, Elinav et al. (2011) demonstrated that oral dysbiosis (imbalances in commensal microbiome) is likely characterized by greater proportions of certain bacterial genera, including *Prevotella*, *Veillonella*, and TM7, which elicit an inflammatory response in the gingival mucosa. Furthermore, several studies showed that *Veillonella* species accounted for a large proportion of the microbiome in caries−affected subjects (Agnello *et al*., 2017 ; Arif *et al*., 2008 ; Chalmers *et al*., 2008 ; Jiang *et al*., 2016). It is known that oral infectious disease results in part from poor oral hygiene. Our findings support these reports, which suggests that *Veillonella* species might be useful in the clinic to treat or prevent oral infectious diseases.

Because it is difficult to discriminate the species of *Veillonella* using 16S rRNA , the distribution and frequency of oral *Veillonella* species in the same saliva samples were examined at the species level by one−step PCR (Mashima et al., 2016) to clarify the oral *Veillonella* species that increase in a poor oral hygiene state. The prevalence of *V. rogosae* decreased as oral hygiene quality decreased, and the detection rate was 73%, 69%, and 58% in the Good, Moderate, and Poor oral hygiene groups, respectively, and *V. rogosae* was the predominant species in all groups. In contrast, the detection rate of *V. parvula* increased significantly as oral hygiene quality decreased, and the detection rate was 6%, 7%, and 17% in the Good, Moderate, and Poor oral hygiene groups, respectively (Fig. 4). These results suggest that changes in the detection rate of some oral *Veillonella* species, such as decreased *V. rogosae* and increased *V. parvula*, can be useful as a biomarker for deteriorating oral hygiene in children (Fig. 4) (Theodorea et al., 2017).

8) Novel Oral *Veillonella* species

As mentioned above, although 13 species of genus *Veillonella* have been established, only five species *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, and *V. rogosae* have been isolated from human oral cavities as oral *Veillonella*
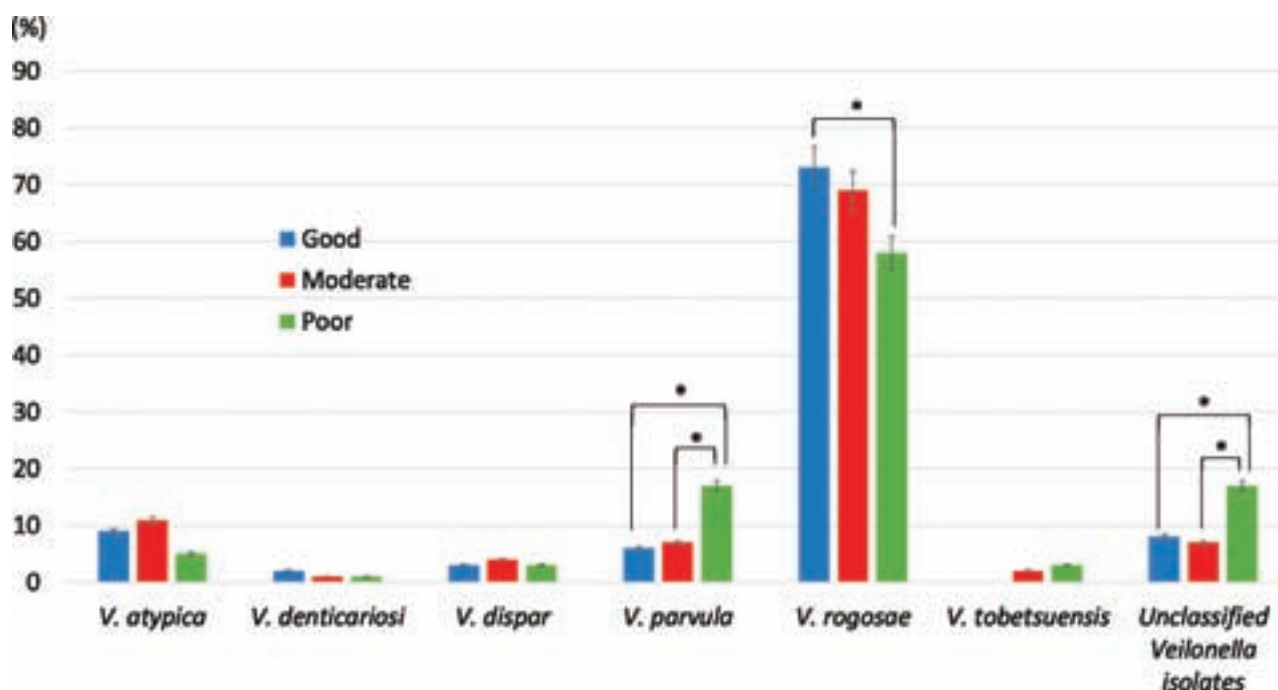

Fig. 4 : Percentages of *Veillonella* species isolated from saliva samples of the Poor, Moderate, and Good oral hygiene groups. Significant differences were evaluated using the Kruskal−Wallis H−test post hoc Mann−Whitney U−test with Bonferroni correction ($P <$ 0.05). Error bars Iadicate 95% confidence intervals.

species, for a long time.

In 2013, Mashima et al. (2013) isolated *V. tobetsuensis* from human tongue biofilms of young adults as a novel *Veillonella* species (Mashima et al., 2013). And it was demonstrated *V. tobetsuensis* was clearly different from the 13 established *Veillonella* species in the *rpoB* and *dnaK* sequence comparison, but were indistinguishable in the 16S rRNA gene sequence analysis. In addition, the draft genome of the *V. tobetsuensis* type strain was sequenced by Illumina HiSeq with sequencing runs for paired–end sequences (Mashima & Nakazawa, 2015). In the study, it was indicated that the genome sequence of the strain was 2,161,277 bp, and contained 1,913 coding sequences, 48 tRNAs, and 3 rRNAs. The study suggested that some protein–coding genes contributed to biofilm formation with oral *Streptococcus* species (Mashima & Nakazawa, 2015).

After 2015, some studies suggested that many novel *Veillonella* species inhabit human oral cavities. For example, Mashima et al. (2015) isolated 442 strains of *Veillonella* from periodontal pockets of 18 subjects (Mashima et al., 2015). Forty–three strains of the 442 could not be identified

from six oral *Veillonella* species already reported by species –specific PCR primers (Igarashi et al., 2009, Mashima & Nakazawa, 2013). In addition, they isolated 101 *Veillonella* strains from tongue biofilms of 10 subjects from 89 Thai children (Mashima et al., 2016). In the study, 61 of the 101 strains were identified as either *V. atypica*, *V. dispar*, *V. rogosae*, or *V. tobetsuensis*. Forty strains had no PCR products with one–step PCR (Mashima et al., 2016), but these 40 strains had positive PCR products with *Veillonella* genus –specific primers. The representative 11 strains in the 40 unknown strains formed distinct taxa with robust bootstrap values in the phylogenetic tree constructed with the *rpoB* gene sequence. Therefore, the authors suggested that there were at least three novel *Veillonella* species in the 40 unknown strains based on the evolutionary trees of the *rpoB* gene (Fig. 5). In the future, *Veillonella infantum* will be proposed as a novel *Veillonella* species for some strains of those 11 representative strains.

Theodorea et al. (2017) isolated 1,609 strains from saliva samples of 107 Thai children and samples were assigned as members of genus *Veillonella* using *Veillonella* genus–spe-
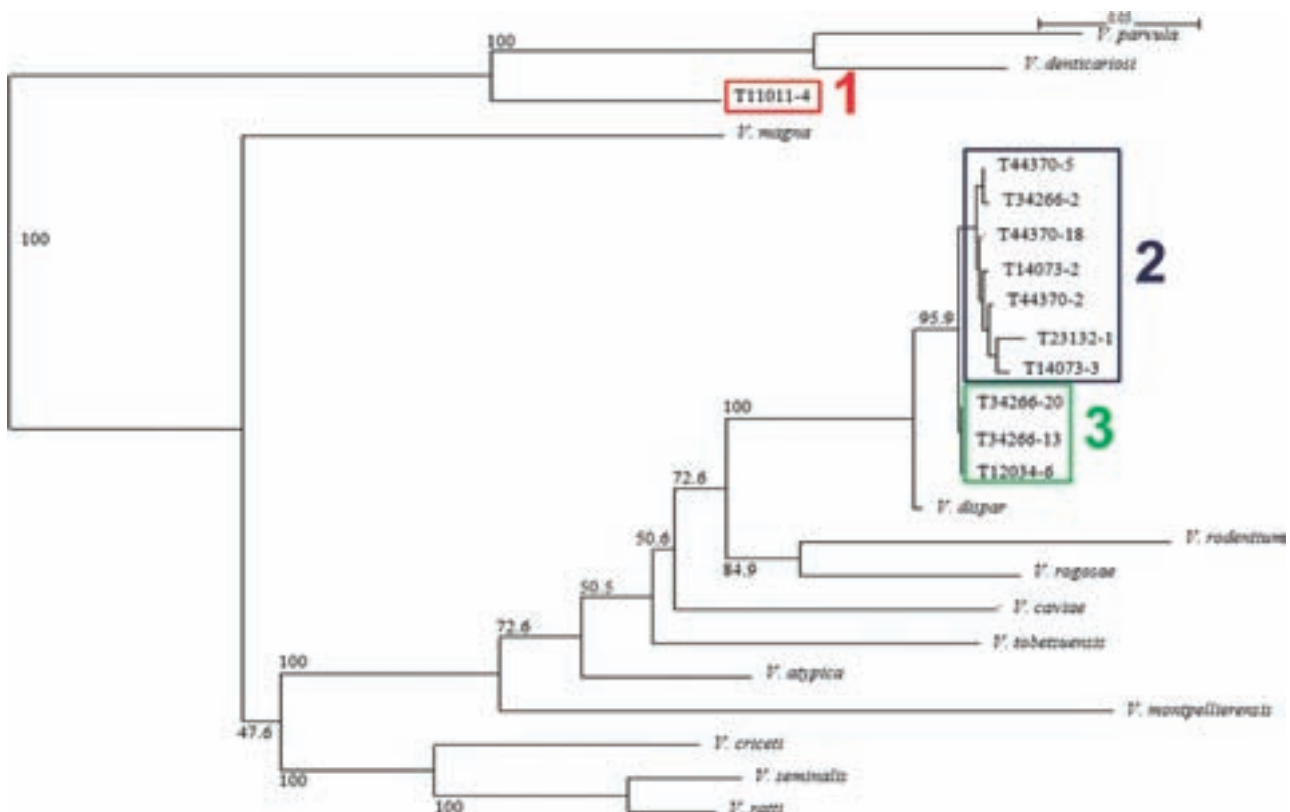

Fig. 5 : Phylogenetic tree based on *rpoB* gene sequences of 11 unclassified strains from tongue biofilms of Thai children and the type strains of 13 established species of *Veillonella*.
Three distinct lineages suggested novel species within *Veillonella*. Bar indicates phylogenetic distance. The number of corners indicates the bootstrap values.

cific PCR primers (Theodorea et al., 2017). One−hundred sixty−seven strains were not classified to any oral *Veillonella* species, but 1442 of the 1609 strains were identified as one of the six oral *Veillonella* species by one−step PCR (Mashima et al., 2016). Subsequently, they proposed several novel species of genus *Veillonella* in human saliva based on phylogeny with sequence analysis of *rpoB* genes of the representative 23 strains in the 167 unclassified strains (Theodorea et al., 2017) (Fig. 6).

Conclusion

Oral *Veillonella* should be investigated to prevent oral infectious diseases caused by oral biofilms. Dr. Morrison Rogosa (National Institute of Dental Research, Bethesda, USA), Dr. David Beighton (Dental Institute, King's College London, England, UK), Dr. Paul E. Kolenbrander (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, USA), and their laboratory team studied oral *Veillonella*. However, there is still little understanding of the actual mechanisms of *Veillonella*. Although we outlined the latest findings on oral *Veillonella* species in this article, the number of studies on oral *Veillonella* remains small. This review will help promote increased research on oral *Veillonella*.
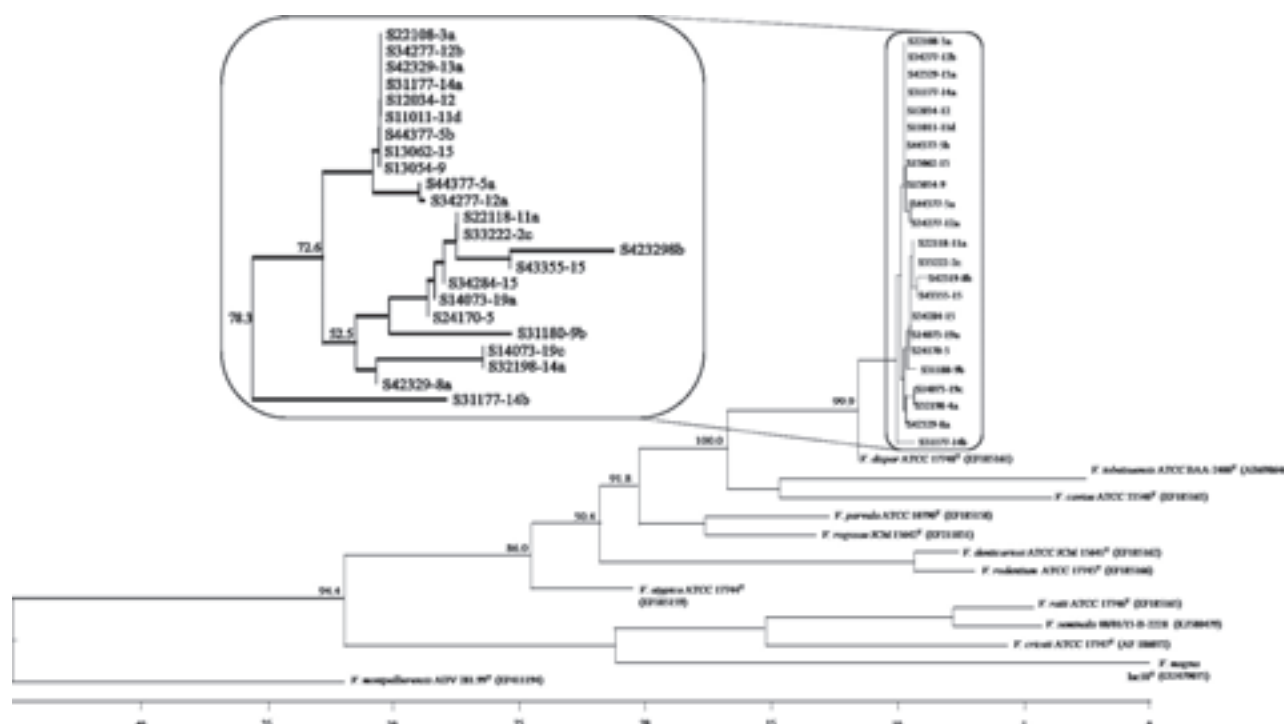
Fig. 6 : Phylogenetic tree based on *rpoB* gene sequences of the 23 representative unclassified strains from the saliva of Thai children and the type strains of the 13 established species of *Veillonella*.
A distinct cluster suggested a novel species within *Veillonella*. Bar indicates the phylogenetic distance. The number of corners indicates the bootstrap values.

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