Prospects of Novel Species of Oral *Veillonella* in Human Saliva

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Abstract

Although 13 species of bacteria have been established in the genus *Veillonella*, only 6 have currently been isolated from human oral cavities (*V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*). Oral *Veillonella* have played a central role as early colonizers that establish multispecies oral biofilms that cause many oral infectious diseases in humans such as periodontitis and dental caries. Our previous study isolated 1,609 strains (confirmed by PCR with the genus-specific primer set) of the genus *Veillonella* from saliva samples of 107 Thai children. Although 167 strains could not be classified under any oral *Veillonella* species, 1,442 strains were linked to the 6 oral *Veillonella* species by PCR with species-specific primer sets. To reveal the phylogenetic characteristics of these unclassified *Veillonella* strains, PCR-based amplifications and sequence analyses of *rpoB* genes were performed for 23 representative strains. When the phylogenetic tree was examined based on the obtained sequence data, these 23 strains formed a cluster distinct from the 13 established species of the genus *Veillonella* with a robust bootstrap value. Thus, the phylogenetic study demonstrated the prospects of several novel species of oral *Veillonella* in human saliva.

Keywords: Novel species; Oral biofilm; Oral *Veillonella*; Phylogenetic tree; *rpoB* gene

Introduction

The genus *Veillonella* consists of small, strictly anaerobic, gram-negative cocci lacking flagella, spores, and capsules that are characterized by their ability to obtain energy from short-chain organic acids [1,2]. Although 13 species have currently been established in the genus *Veillonella*, only 6 species (*V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*) have been isolated from human oral cavities [2-9]. The main habitats of the oral *Veillonella* are tongue biofilms, dental biofilms, buccal mucosa, and saliva [10-12]. Oral *Veillonella* has been found in severe early childhood caries [13], apical root canals [14], and dental tubules [15]. Additionally, oral *Veillonella* are also predominantly found in saliva [16] and subgingival biofilm specimens [17] from patients who have chronic periodontitis.

It was reported that oral *Veillonella* play a central role as early colonizers that establish multispecies oral biofilm communities [18]. Oral biofilms are known to cause many oral infectious diseases, such as periodontitis and dental caries in humans [2,19]. It was reported that oral *Veillonella*, found throughout the entire oral cavity, comprise as much as 10% of the bacterial community initially colonizing the enamel [20, 21], and often form biofilms with the *Streptococcus* species [22]. Some studies have also shown that during the formation of early dental plaque, the ratio of *Veillonella* to *Streptococcus* species varied in mixed-microbial colonies [23].

The identification of *Veillonella* isolates at the genus level is relatively simple, but identification at the species level remains difficult because of the lack of conventional phenotypic and biochemical tests [24]. However, molecular methods based on the 16S rRNA gene sequencing have previously been used to identify *Veillonella* strains at the species level [25,26]. Unfortunately, recent studies have demonstrated that using 16S rRNA gene sequencing exclusively for identification purposes is unreliable because of the high levels of sequence conservation.
in the 16S rRNA gene sequence among several Veillonella species [5,8,9,25]. To overcome this difficulty, Beighton et al. [27] used the rpoB gene sequence to identify isolates of the genus Veillonella from the human tongue at the species level. Furthermore, our previous study established the first successful one-step PCR method using species-specific primer sets based on the highly variable region of the rpoB gene of 6 species of oral Veillonella (positions 2,500-3,100) [12]. This one-step PCR method is easier and more effective than the previous molecular methods described above in identifying oral Veillonella at the species level.

Our previous study examined the distribution and frequency of oral Veillonella species in the saliva of 107 Thai children with varying oral hygiene statuses [28]. PCR with the genus-specific primer sets confirmed 1,609 strains, isolated in the study, as members of the genus Veillonella [27]. Furthermore, 1,442 strains were identified by the one-step PCR method as V. atypica, V. denticiarossi, V. dispar, V. parvula, V. rogasae, or V. tobetsuensis. However, 167 strains could not be classified into any oral Veillonella species. This short communication seeks to report the phylogenetic position and diversity of 23 of the 167 unclassified Veillonella strains by phylogenetic analysis with the rpoB gene sequence.

23 unclassified Veillonella strains-S22108-3a, S34277-12b, S42329-13a, S31177-14a, S12034-12, S11011-11d, S44377-5b, S13062-15, S13054-9, S44377-5a, S34277-12a, S22118-11a, S33222-2c, S42329-8b, S34355-15, S34284-15, S14073-19a, S24170-5, S31180-9b, S14073-19c, S32198-4a, S42329-8a, S31177-14b-were selected as representative strains and cultured in BactoTM Brain Heart Infusion (Difco Laboratories, BD) broth supplemented with 5% (v/v) defibrinated sheep blood (BHI agar), hemin (10μg/mL), and menadione (5μg/mL) under anaerobic conditions with 80% N2, 10% CO2, and 10% H2 at 37 °C for 5d.

Genomic DNA was extracted from individual bacterial cells using an Insta Gene Matrix Kit (Bio-Rad) and PCR-based amplification and sequence analyses of rpoB gene was performed using previously described primers [27]. The sequences were determined by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and were aligned and connected by using SEQMAN Pro of the LASERGENE 14 program (DNASTAR). The programs MEGALIGN including CLUSTAL W using the neighbor-joining (NJ) method [29] were used to compare sequences and to reconstruct an evolutionary tree. The rpoB sequences of the 23 unclassified Veillonella strains were aligned against the sequences of the representative Veillonella type strains retrieved from GenBank.

Alignment of the rpoB gene sequences showed 97.6-100% similarity among the sequences of the unclassified 23 Veillonella strains. These results indicate that these 23 strains are closely related. The evolutionary tree obtained for the type strain of the established species of genus Veillonella and the unclassified 23 Veillonella strains is shown in Figure 1. Based on the tree, the 23 unclassified Veillonella strains formed a cluster distinct from the established Veillonella species. Although the most closely related species was V. dispar, the robust bootstrap value of 99.9% indicated that these 23 strains formed a taxon distinct from V. dispar.

![Figure 1: Neighbor-joining tree-based analysis of the rpoB gene sequences showing the relationship between the 23 unclassified Veillonella strains and the type strains of the established species of genus Veillonella. Accession numbers for rpoB gene sequences are given for each established strain. Bootstrap values are indicated at corresponding nodes. Nucleotide Substitution per 100 residues.](image)

**Conclusion**

This study demonstrated prospective novel oral Veillonella species in human saliva. It is expected that the new perspective of oral biofilm communities at early stages would be investigated by this discovery of novel Veillonella species.

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