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Investigation of in Vitro Mineral Forming Bacterial Isolates from Subgingival Calculus

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Abstract

The mechanism that dictates the formation of dental calculus, an important component of periodontal health, has yet to be sufficiently explained. The effect of bacterial flora on dental calculus formation is important for periodontal status. Therefore, this study aims to investigate the mineral forming bacteria from subgingival calculus under anaerobic conditions.

Bacteria have been isolated from subgingival calculus, and isolates were examined for mineralization under anaerobic conditions. Bacterial isolates capable of crystal formation on plates were identified by their 16S rDNA sequences.

Bacterial isolates from subgingival dental calculus that form minerals in vitro have been identified as Streptococcus spp.

This is the first report to identify and show that bacteria from subgingival calculus under anaerobic conditions are involved in the formation of dental calculus.

Key words: *Subgingival dental calculus, streptococcus, biomineralization, 16S rDNA sequence*

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Introduction

Bacterial plaque is the primary etiological factor affecting the development and progress of periodontal disease, particularly because bacterial plaques are commonly accompanied by the presence of dental calculus [1, 2]. As well as forming a reservoir for toxic bacterial products and antigens, the dental calculus provides a porous environment for the retention and growth of the bacterial plaque and is a predisposing factor for the development and progression of periodontal disease [3,4]. Periodontal breakdown is associated with the presence of dental calculus, and an increasing dental calculus may increase the rate of damage that is related with plaque bacteria [1,4,5]. Subgingival [6] and supragingi-

val [7] calculus formation is associated with the formation of chronic inflammatory periodontal disease. Once formed, the presence of dental calculus may be inhibited by natural and mechanical oral hygiene, and it influences and enhances the growth of the pathogenic plaque [8].

Formation of calculus always appears due to the formation of the bacterial plaque, which produces the calcified plaque and an inorganic matrix. Following the tooth eruption or dental prophylaxis, various bacteria adhere to the enamel pellicle (adherence) [7]. In the mineralization of the dental plaque, calcium salt deposits or crystallization develop primarily on the intercellular matrix and bacterial surfaces and then within the bacteria [9]. Calcifying oral bacteria were determined to be the initiator of mineralization in the tooth-gingiva junction [10]. Formation or integration of the calculus is associated with the bacterial composition of the plaque and the interaction between the bacteria and the environment [11]. In parallel with previous in vivo studies, in vitro experiments show that Corynebacterium (Bacterionema) matruchotii, Streptococcus mutans, Actinomyces spp., and Candida albicans are associated with mineralization in different intra- or extra- cellular cases [11,12].

Considering the composition of the dental calculus, especially the phosphate salts of the calcium, this is an area of great interest. The formation of calcium compounds of bacteria in different ecosystems has been intensively studied, with some underlying mechanisms being suggested [11,13,14]. In vitro experiments using some bacteria were performed to explain the mechanism of the calculus formation. It was shown that Streptococcus salivarius and S. sanguinis increase the dental pH due to their arginine deaminase activity that generates urea and ammonium compounds [15]. Bacteria in the plaque increase the local pH; and when the pH increases, calcium-phosphate ions collapse [9]. Several experiments conducted with oral bacteria were performed in environments containing highly intensive ions and therefore they have remained quite limited. Even though the supragingival calculus is associated to pH changes, buffering systems, calcium sources (salivary), food (nutritional residues), microbial diversity, and abundance (facultative anaerobes and aerobes) [4,7,16], the steps leading to formation of subgingival calculus remain unclear.

The aim of this study is to isolate and identify the mineral forming bacteria from subgingival calculus under anaerobic conditions. The features of the isolated bacteria that contribute to mineral formation in pure and complex cultures were investigated. The main purpose of the study is to provide a new direction for studies investigating the formation of the dental calculus.

Materials and Methods

Collection of Samples

Subgingival calculus samples were collected from 3 patients with periodontal disease (Atatürk University, Ethics Committee of Dentistry Faculty approval, 2011/10) at the Department of Periodontology, Faculty of Dentistry, Atatürk University, as part of their conventional treatment. The collected samples were transfered to laboratory in phosphate buffer (pH 7.2) for bacterial isolation.

Bacterial Isolation and Growth Conditions

The samples were shaken in buffer (phosphate buffer pH 7.2) for one night, then cultured colonies were grown in B4 media (glucose 10 g/L, yeast extract 4 g/L, calcium acetate 2.5 g/L, and agar 18 g/L pH 7.2) [17], modified BHIA (BHIA 39 g/L, KH2PO4 2 g/L, and CaCl2 0.8 g/L pH 7.2) and B2 (glucose 1 g/L, yeast extract 1 g/L, casein 4 g/L, tris (tris(hydroxymethyl) amino methane) 12 g/L, calcium acetate 1.5 g/L, and agar 15 g/L pH 7.4) (modified from Barış [14], Park et al. [18] and Roh et al. [19]) in an anaerobic jar at (37 °C) and under anaerobic conditions (with Anaerobic Gas Pack, Oxoid, BR0038B). Following incubation, in vitro mineralization experiments were performed. Cultures were observed under the light microscope for 3–20 days and positive results were replicated from pure cultures for verification. Isolated and purified bacterial strains were stored in Nutrient Broth containing 15% glycerol at −86 °C for further study [14,20]

Extraction of Genomic DNA

Genomic DNA was extracted from bacteria isolates using a method previously described by Wilson [21]. The bacterial samples were inoculated on thyripticase soy agar plates and incubated overnight at 37 °C. The cells were harvested and suspended in 567 µL of TE buffer with 30 µL of 10% SDS and 3 µL of 20

mg/mL proteinase K. The bacterial suspensions were thoroughly mixed and incubated for 1 h at 37 °C. One-hundred microliters of 5 M NaCl and 80 µl of CTAB-NaCl was added and the mixture was kept at 65 °C for 10 min. Approximately an equal volume (0.7 to 0.8 mL) of chloroform:isoamyle alcohol (24:1) was added and tubes were shaken for 20 s and centrifuged at 12 000 rpm for 5 min. The supernatant was transferred into a fresh tube, an equal volume (0.6–0.7 mL) of phenol:chloroform:isoamyle alcohol (25:24:1) was added and the tube was shaken for 20 s and centrifuged at 12 000 rpm for 5 min. The supernatant was transferred into a fresh tube and the genomic DNA was precipitated with isopropanol. The pellets were washed with 70% ethanol three times, dried, and suspended in 50 µL of TE buffer. The purity of the DNA was determined using a spectrophotometer and the A260 and A280 values and stored at –20 °C until further use [14,21].

PCR Amplification and Sequencing Analysis

The amplification reaction mixture was prepared in a 30 µL volume containing 3 µL 10× PCR buffer, 0.6 µL dNTP mixture (10 mM of dATP, dGTP, dCTP, and dTTP, Sigma-Aldrich Co., USA), 0,3 µL each primer (5 µM) (27f 5′-AGA GTT TGA TCC TGG CTC AG-3′; 1492r 5′-GGT TAC CTT GTT ACG ACT T-3′), 1.8 µL MgCl2 (25 mM), 1.2 µL DMSO (20X), 0.3 µL Taq DNA polymerase (5 unit/µL, Sigma-Aldrich Co., USA), 21.5 µL sterile ddH2O, and 1 µL genomic DNA.

The reactions were performed in a thermal cycler (Corbett Research CG1-96, Australia) without mineral oil. PCR master mix with ddH2O (instead of genomic DNA) was used as negative control. After an initial denaturation at 95 °C for 2 min, the PCR profiles were set as follows: 1 min of denaturation at 94 °C, 1 min of annealing at 58 °C, and 1 min extension at 72 °C, for 35 cycles, and a final extension at 72 °C for 7 min. The samples were analyzed by electrophoresis on a 1% agarose gel and then stained with ethidium bromide (0.5 µg/mL). The PCR product bands were photographed under ultraviolet light [14,22].

PCR products were sequenced by Macrogen Inc. (Macrogen, Korea). Sequences were edited with the BioEdit program (Ibis Biosciences, CA, USA) and compared for similarities with the nucleotide sequences in the NCBI library [22,23].

Results

Bacterial Isolation and Biomineralization

The bacterial colonies from subgingival calculus were inspected under the light microscope on the days 3, 5, 7, 10, 15, and 20 and the crystal forming colonies were transferred to new media. Each purified bacteria was monitored until day 20 and the experiments were repeated for the bacteria that exhibited crystallization.

Six isolations were performed in B4 media, but because no crystallization was observed, this medium was not used in the remainder of the study. The medium that gave the best results for bacterial growth was modified BHIA. Although this medium did result in the formation of colonies, one bacterium developed fewer crystals than the rest; therefore, this medium was not used in subsequent experiments. We used B2 media in this study, which supported the formation of a larger colony number of the proper size of crystals. Biomineralization experiments were sustained by transferring 34 colonies to a new medium; each colony was thought to be different in the B2 medium. Each biomineralization experiment using the 34 pure bacterial cultures was repeated. Of the initial 34, 16 isolates could form crystals (Figure 1).

Table 1. Identification results of mineral forming bacterial isolates, which were obtained in the subgingival calculus.

Codes of Isolate	Names of Organism	Accession number
D1	Streptococcus mutans	KC505240
D2	Streptococcus mutans	KC628748
D3	Streptococcus anginosus	KC628749
D4	Streptococcus gordonii	KC628750
D5	Not detected	-
D6	Streptococcus sanguinis	KC628751
D7	Streptococcus gordonii	KC628752
D8	Streptococcus gordonii	KC628753
D9	Streptococcus constellatus	KC628754
D10	Streptococcus constellatus	KC628755
D11	Streptococcus sanguinis	KC628756
D12	Streptococcus sanguinis	KC628757
D13	Streptococcus anginosus	KC628758
D14	Not detected	-
D15	Streptococcus massiliensis	KC628759
D16	Streptococcus gordonii	KC628760

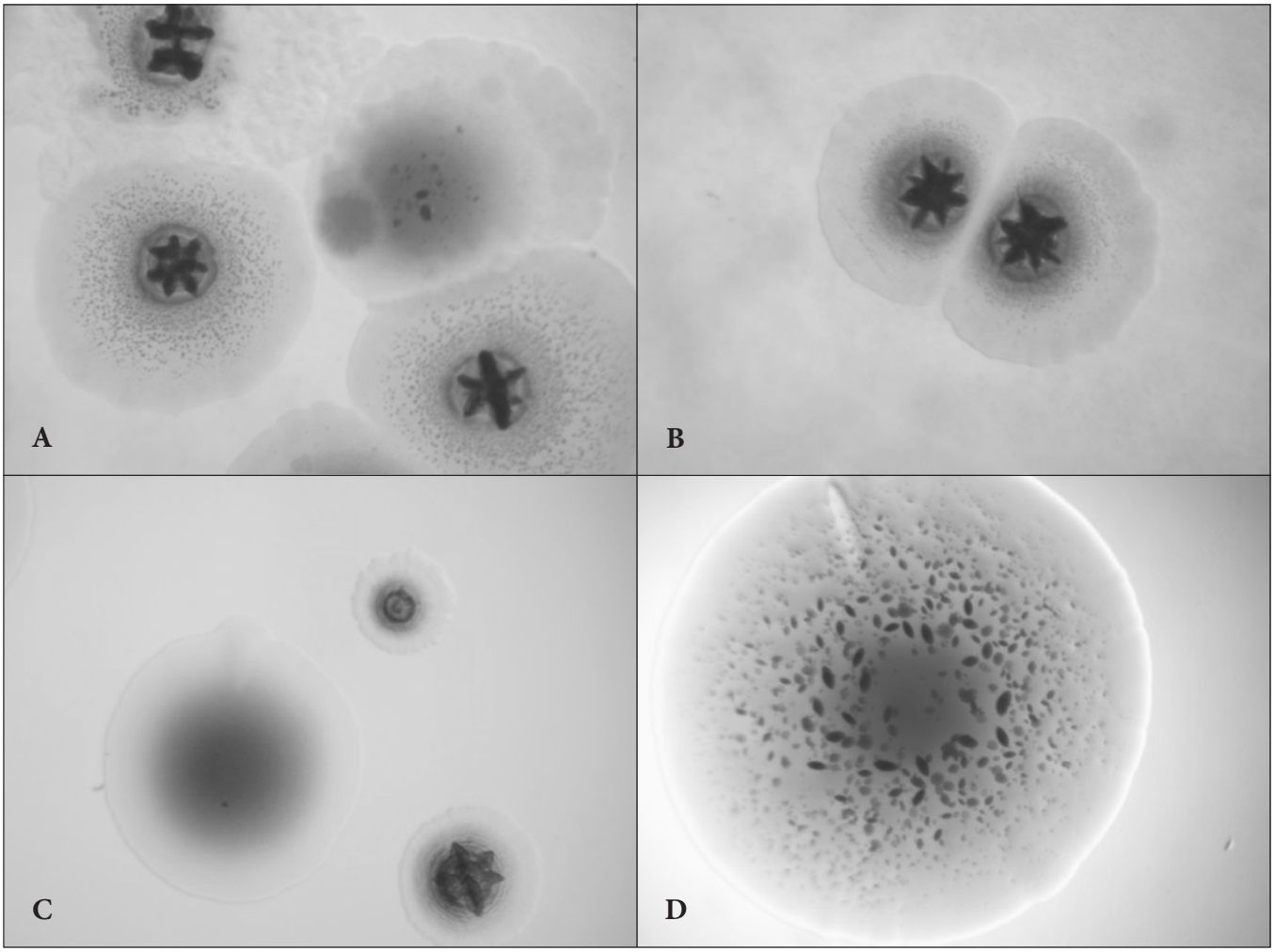

Figure 1. Crystal formations in pure and complex cultures in the B2 growth media. **A – C)** mixed culture; **B – D)** different types pure cultures growth and cristalization.

Identification of Bacterial Isolates (by Sequence Analysis)

Sixteen bacterial isolates, which formed crystals in B2 media, were grown under anaerobic conditions and DNA was isolated. The amplification of the DNA with 16S rRNA primers produced a single amplicon per sample with sizes of 1400–1500 bp on the gel. The obtained amplicons were sent to Macrogen for DNA Sequence analysis. The sequencing results were analyzed using BioEdit and compared by using the BLAST program and the Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Table 1 shows the obtained results and accession numbers.

Discussion

The calculus is a mineralized dental plaque whose inorganic components are surrounded with a non-mineralized bacterial plaque and mainly consist of calcium phosphate salts (e.g., dicalcium phosphate dehydrate, octacalcium phosphate, hydroxyapatite, and magnesium-tricalcium phosphate) (Whitlockite) [2,5]. Even though the first phases of dental plaque formation (bacterial adhesion, accumulation and proliferation) can partially be explained [24], changes to the dental plaque bacterial flora have yet to be fully understood [25]. It is known that flora can play a part in the maintenance of homeostasis; however, some of these bacteria might cause diseases when homeostasis is disrupted [26]. For example, it is known that S. mutans, which is commonly found in dental plaque, can cause tooth decay under some conditions [27]. Importantly, bacteria directly influence the pH level of the mouth. Some bacteria on the dental plaque produce ammoniac by urea hydrolysis, which makes the pH in the mouth mildly alkaline, optimal for calculus formation [5,28]. The increase or decrease of mouth pH can result in mineralization or demineralization, respectively. This occurs due to fluctuating calcium and phosphate levels of tooth surface and saliva and/or plaque [29].

The collapse of calcium phosphate salts causes mineralization and, as a consequence, the formation of calculus, a secondary factor for the development of the periodontal disease. The initial studies conducted to determine the mechanisms of bacterial mineralization were performed on Bacterionema (Corynebacterium) matruchotii [30], and it was concluded that phospholipid fractions form apatite in the interior [31]. In order to understand the nature of the calculus—as well as to explore bacterial mineralization—some groups have investigated the bacterial contents of the calculus. Living aerobic and anaerobic bacteria were identified on the supragingival calculus (especially in interdental canals and lacunas) collected from patients with moderate and severe periodontitis [32]. On the subgingival calculus, the periodontal pathogens Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Treponema denticola were identified in deeper cavities of the lacuna and canals [33]. Complex lipids produced by P. gingivalis were found on lipid extracts obtained from subgingival calculus [34].

The essential goals of this study were to investigate how bacteria affect mineralization and dental plaque formation as well as to address their effects on calcification. The bacterial contents of the calculus do not indicate whether these bacteria are calcifying nor does it show the roles of bacteria on calculus etiopathology.

The medium selection is critical to conduct the mineralization studies. The medium used should allow for the isolation and formation of minerals. In these experiments, the media used made it difficult to isolate bacteria due to its high calcium content and buffered environment [17,20]. However, we were able to generate a medium that allowed for the observation of mineral formation from the obtained isolates. The B2 medium was designed based on previous literature [14,18-20,35] and the crystal formation was observed from day 3. However, this medium is not sufficient to culture most microorganisms that are present in the mouth and dental calculus flora. This probably explains why bacteria such as A. actinomycetemcomitans, P. gingivalis and T. denticola, which were previously identified on the calculus [11,12], could not be isolated in this study. Another reason why all microorganisms in this study were Streptococcus is that only these isolates could form minerals. The presence of Gram-positive bacteria in the mineralized calcium compounds is common [17]. In addition, other Streptococcus types in calcification, which are common oral flora bacteria, are rare. Streptococcus types are expected to be active during demineralization and tooth decay due to their fermentation capability, from which organic acids are produced; these, once released in the medium, decrease pH and deteriorate the enamel (calcifying structure) [36-39]. However, because the medium used in the study was buffered with calcium and tris, it enabled mineralization. Streptococcus species do not utilize urease activity because they require CO2 [36,40,41]. Thus, it is thought that they can participate in the calcification by adhering to cell surface compounds, which is required for calcium precipitation and mineralization [42]. Considering the in vivo conditions in the subgingival area (such as other microorganisms, anaerobic medium, immune system, gingival crevicular fluid, calcium density, and pH), the previously identified in vitro mechanism remains valid [43,44].

Since several Streptococcus types colonize both hard and soft tissues, they could encounter the oral flora and also infect various parts of the human body [27,45]. Streptococci are abundant on the hard tissue, coexisting with S. gordonii and S. mutans; and S. gordonii and S. sanguinis are leading causes of plaque formation [46]. S. mutans is the most frequently isolated bacteria from the oral flora, the most common bacteria on decaying teeth, and found in atheromatosis plaques of the cardiac valve. S. mutans was found to be associated under different inter- and/or extra- cellular calcification conditions [11,47]. S. sanguinis was observed with increased pH, and this condition correlates with its effect on mineralization [29]. Members of the S. milleri group, S. anginosus, and S. constellatus, were found to be associated with purulent infections and not with mineralization [15]. In addition, S. constellatus formed denser colonies on and adhered more frequently to hydroxy apatite than they did with titanium [48]. There is a limited number of studies on S. massiliensis [49], one of the bacteria identified in this work. In particular, this is likely the first time the microorganism has been isolated from the oral flora.

To our knowledge, this is the first report on the abil-

ity of Streptococcus types to form minerals in vitro under in anaerobic conditions. We also saw oral colonization of the S. massiliensis type, which has recently been isolated from the blood and infected parts of patients. Our results support the idea that bacteria have a potential role in the etiopathology of the subgingival calculus.

Consequently, the data not only present various explanations for the roles of bacteria in the subgingival area, but also raise several new questions requiring further study.

Conflict of interest statement

We do not have any financial support or conflict of interest for this paper.

Ethical approval

The study was approved by the Ethics committee of Atatürk University, Ethics Committee of Dentistry Faculty approval, 2011/10.

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