Original Article

Investigation of *In vitro* Mineral Forming Bacterial Isolates from Supragingival Calculus

O Baris, T Demir[1], M Gulluce

Department of Biology, Faculty of Science, Atatürk University, [1]Department of Periodontology, Faculty of Dentistry, Atatürk University, Erzurum, Turkey

Aim: Although it is known that bacterial mechanisms are involved in dental calculus formation, which is a predisposing factor in periodontal diseases, there have been few studies of such associations, and therefore, information available is limited. The purpose of this study was to isolate and identify aerobic bacteria responsible for direct calcification from supragingival calculus samples. **Materials and Methods:** The study was conducted using supragingival calculus samples from patients with periodontal disease, which was required as part of conventional treatment. Isolations were performed by sampling the supragingival calculus with buffer and inoculating the samples on media on which crystallization could be observed. The 16S recombinant DNA of the obtained pure cultures was then amplified and sequenced. **Results:** A few bacterial species that have not previously been associated with mineralization or identified on bacterial plaque or calculus were detected. The bacteria that caused mineralization an aerobic environment are identified as *Neisseria flava*, *Aggregatibacter segnis*, *Streptococcus tigurinus*, and *Morococcus cerebrosus*. **Conclusion:** These findings proved that bacteria potentially play a role in the etiopathology of supragingival calculus. The association between the effects of the identified bacteria on periodontal diseases and calculus formation requires further studies.

KEYWORDS: *Bacteria, biomineralization, supragingival dental calculus*

INTRODUCTION

The primary factor involved in the formation and progress of periodontal diseases, which are the main cause of tooth loss in adults, is bacterial plaque. The mineralized form of bacterial plaque is known as calculus.[1,2] Periodontal degradation is explicitly associated with the presence of calculus; abundant calculus might increase the rate of damage that is associated with plaque-forming microorganisms.[3,4] The reason for the increase in damage is that the calculus facilitates further bacterial involvement by forming a rough surface for colonization, and therefore serves as a predisposing factor in pocket development.[4] The nonmineralized net of islands and canals in the supragingival calculus provides a suitable environment for the expression of virulence factors by possible periodontopathogens.[4] In addition, mineralized calculus is always covered with a living nonmineralized bacterial layer that is metabolically active.[5]

The amount of calculus is affected by a number of variables including age, sex, ethnic origin, diet, localization in the oral cavity, oral hygiene, bacterial composition, host response differences, access to professional hygiene services, mental and physical barriers, and systemic diseases.[3] The composition of the bacterial colony in the oral cavity is important in calculus formation because bacteria have a direct effect on the pH level in the mouth.[3,6]

Address for correspondence: Prof. T Demir,
Department of Periodontology,
Faculty of Dentistry,
Atatürk University, Erzurum 25240, Turkey.
E-mail: turgdemir@hotmail.com

Access this article online	
Quick Response Code:	**Website**: www.njcponline.com
	DOI: 10.4103/1119-3077.187316

The calcium salts present in the calculus are of interest in this respect. The mechanisms by which calcareous crystals are formed are similar among species. Extensive studies of bacteria from different ecosystems have identified a few possible mechanisms.[7,8] To explain the mechanism of calculus formation by bacteria, *in vitro* experiments have been conducted and have shown that *Streptococcus salivarius* and *Streptococcus sanguinis* increase dental pH due to arginine deaminase activity, which leads to the formation of urea and ammonia compounds.[4,6] *Corynebacterium* (*Bacterionema*) *matruchotii*, *Streptococcus mutans*, *Actinomyces* spp., and *Candida albicans* have also been associated with mineralization under different intra- or extracellular conditions.[7] As a result, the bacteria in the plaque increases the local pH, and when the pH increases the calcium-phosphate ions collapse, which is the natural result of a physical rule.[6] Many experiments have been performed with oral bacteria in environments with high ion levels, and thus, their findings have very limited applications.

Although it is partially possible to explain the formation of supragingival calculus by considering data such as buffering systems and calcium resources (saliva), pH changes, food (nutritional residues), and microbial diversity, and burden (facultative anaerobes and aerobes), it still remains unclear which bacterial species are directly responsible for calcification.

This study aimed to isolate and identify bacterial species capable of mineralization from the supragingival calculus under aerobic conditions. We also investigated the mineralization ability of the isolated bacteria in pure and mixed cultures. In light of the data obtained, the prevention and/or deceleration of calculus formation are also discussed.

MATERIALS AND METHODS

Collection of samples

Supragingival calculus samples were collected from 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 transferred to laboratory in phosphate buffer (pH 7.2) for bacterial isolation.

Bacterial isolation and growth conditions

The samples were inoculated into a buffer (phosphate buffer pH 7) and kept a shaker overnight. The bacterial colonies were cultivated 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),[9] modified Mitis Salivarius Agar (MSA 35 g/L, $CaCl_2$ 1 g/L pH 7.2, and sterile 1% solution of potassium tellurite 10 ml/L), 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)[8,10] at 37°C. Cultures were observed under the light microscopy for 7–40 days, and positive results were repeated as pure cultures for verification. Isolated and purified bacterial strains were stored in nutrient broth containing 15% glycerol at −86°C for further studies.[8,11]

Extraction of genomic DNA

Genomic DNA was extracted from bacteria isolates using a method previously described by Wilson.[12] The purity of the DNA was determined using a spectrophotometer and the A_{260} and A_{280} values and stored at −20°C until further use.[8,12]

Polymerase chain reaction amplification and sequencing analysis

Amplification reaction mixture was prepared in a 30 μL volume containing 3 μL 10× polymerase chain reaction (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 $MgCl_2$ (25 mM), 1.2 μL DMSO (20×), 0.3 μL *Taq* DNA polymerase (5 unit/μL, Sigma-Aldrich Co., USA), 21.5 μL sterile ddH_2O, and 1 μL genomic DNA.

The reactions were performed in a thermal cycler (Corbett Research CG1-96, Australia) without mineral oil. PCR master mix (without genomic DNA) and ddH_2O were 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.[8,10]

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.[10,13]

RESULTS

Bacterial isolation and biomineralization

Bacterial colonies isolated from the supragingival calculus samples were observed under light microscopy on days 7, 10, 15, 21, and 40, and the crystal forming
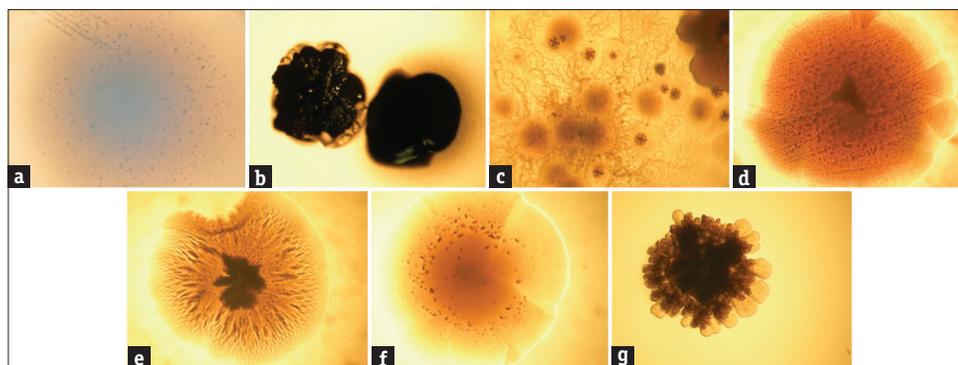
Figure 1: Crystal formations in pure and complex cultures in the growth media. (a) B4 medium (D18); (b) modified Mitis Salivarius Agar medium left crystallized (D21) and right normal colony; (c) mixed culture in the B2 medium; (d) D20, (e) D22, (f) D18, (g) D19 different types pure cultures growth and crystallization in the B2 medium

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

Isolates	Bacterial name	Similarity	A number of bases	Accessions number
D18	*N. flava*	100	1356	KF261347
D19	*A. segnis*	99	699	KF261348
D20	*S. tigurinus*	100	1391	KF261349
D21	*S. tigurinus*	99	1391	KF261350
D22	*M. cerebrosus*	99	996	KF261351

N. flava=*Neisseria flava*; *A. segnis*=*Aggregatibacter segnis*; *S. tigurinus*=*Streptococcus tigurinus*; *M. cerebrosus*=*Morococcus cerebrosus*

colonies were transferred to new media. The purified bacterial isolates were monitored until day 40, and the experiments were repeated for any bacteria that exhibited crystallization.

Even though 11 isolates were obtained from B4 media crystallization was only observed in 1 sample [Figure 1a], and K116 isolate could not be amplified by PCR. Thus, B4 medium was not used in subsequent parts of the study.

Even though 14 isolates were obtained from the biomineralization experiments that used B2 and modified MSA media; crystallization was only observed in a few. Some isolates were positive in the mixed culture (during isolation); however, no crystallization was observed in pure culture. Considering the colonies and the convenience of observing crystal formation, modified MSA was not used in subsequent experiments due to its dark color and the difficulty in examining stiff colony formations because of crystallization [Figure 1b]. All samples were compared, and five isolates grown in B2 medium that formed crystals in pure culture were selected for sequencing. Figure 1 shows the isolates grown in different mediums. All experiments were replicated for verification.

Identification of bacterial isolates (by sequence analysis)

DNA was isolated from six crystal-forming bacterial isolates from the B2 media. The DNA samples were synthesized with 16S ribosomal RNA primers and formed single bands 1400–1500 bp in size when run on a gel. The amplicons were then sent to Macrogen for DNA sequencing. Since K116 isolate showed poor growth and insufficient DNA was extracted, it could not be sequenced. The sequencing results were analyzed using BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and compared using the Basic Local Alignment Search Tool program and GenBank (http://blast.ncbi.nlm.nih.gov/blast.cgi). Table 1 shows the sequencing results and accession numbers.

DISCUSSION

Dental calculus is due to bacterial biofilm formation, which generates an inorganic matrix that is associated with plaque calcification.[14] The mechanisms by which minerals are precipitated within the biofilm matrix (especially calcium and phosphate) remain a mystery. Regarding calcium-phosphate salts, the supersaturation of saliva (especially the plaque fluid) could act as a propellent for the mineralization of dental plaque. Calculus formation is not only affected by the supersaturation of calcium-phosphate salts but also by a variety of other factors such as the saliva flow rate and inhibitors and supporters of calculus formation.[14] Increases and decreases in pH cause mineralization and demineralization, respectively. These events occur as a result of changes in the calcium and phosphate concentration between the tooth surface and the saliva and/or plaque.[15] The precipitation of calcium-phosphate salts causes mineralization and subsequently forms calculus, which is a secondary factor associated with periodontal disease. The first studies that aimed to determine the bacterial mineralization

mechanism used *Bacterionema* (*Corynebacterium*) *matruchotii*[16,17] and revealed that phospholipid fractions formed apatite. There have been studies not only of direct bacterial mineralization but also of the bacterial community composition in the calculus. Bacteria have been identified from supragingival calculus samples collected from patients with moderate and severe periodontitis (especially in the interdental canals and lacunas).[18] Thus, bacteria that are responsible for direct calcification might coexist with bacteria that are not.

One study that assessed such a possibility revealed for the first time that different *Streptococcus* species that have not previously been associated with calcification *in vitro* in an anaerobic environment were associated with mineralization.[10] Very interesting results were also obtained in the present study conducted under aerobic conditions. This is the first report associating the bacteria reported here with calculus formation. A number of Gram-positive species have been shown to play a role in the precipitation of calcium compounds.[8,10,19] For instance, saliva samples were analyzed to determine the presence of *Streptococcus tigurinus,* which has been associated with calcification and identified as an agent in invasive infections (especially infective endocarditis), but the species was found to be uncommon in the oral area.[20] Calcification is not common among the Gram-negative bacteria and is rarely mentioned except for some soil bacteria in the literature.[9,10]

One bacterium detected in this study, *Aggregatibacter segnis* (*Haemophilus segnis*), is a Gram-negative commensal in the human oropharynx.[21] A very limited number of studies have reported the pathogenicity of this organism. Petsios *et al*.[22] asserted that *A. segnis* was a possible router for periodontal disease. There is no literature regarding the calcification potential of this bacterium, but it is thought to cause mineral accumulation due to its adhesive capabilities and cell structure. The fact that it is an important precessor of dental plaque and can coexist with other bacteria supports this contention.[23,24]

On the other hand, little is known about various *Morococcus* species, which have been associated with calcification. Only 1 isolate of *Morococcus cerebrosus* was reported until recently isolated from a brain abscess (cerebellar).[25] However, a more recent study that investigated the bacterial community associated with the oral cavity (saliva, supra and subgingival plaque, tongue dorsum, hard palate, buccal mucosa, keratinized gingiva, palatine tonsils, and throat), skin (right and left antecubital fossa, right and left retroauricular lines), vaginal area, and nasal and stool samples in 237 individuals reported *M. cerebrosus* in the subgingival

plaque.[26] The same study also detected *Neisseria* sp. in the saliva, subgingival plaque, tongue, tonsils, and throat.[26] *Neisseria flava* was also found in the oral area in our study and was associated with direct calcification. *Morococcus* and *Neisseria* are from the same family, and some publications have asserted that these species produce carbonic anhydrase.[27,28] Even though it is possible to associate mineralization with carbonic anhydrase, more comprehensive studies are required to prove this. *In vivo* interactions (such as those with other microorganisms, saliva, density, and pH), especially in the supragingival area, can be different from *in vitro* results. However, *in vitro* studies provide basic information for further studies by providing primary data for explaining possible mechanisms.[29,30]

CONCLUSION

This study aimed to isolate and identify bacterial species present in the supragingival calculus that can induce mineralization under aerobic conditions and has achieved this purpose. The findings support the notion that bacteria play a potential role in the etiopathology of supragingival calculus, and also identified for bacterial species that have not previously been associated with calcification, calculus, and/or bacterial plaque, but that contribute to *in vitro* mineral formation in an aerobic environment. Future studies based on these findings should be able to determine the relationship between these bacteria and periodontal diseases as well as the extent to which these bacteria are involved in calculus formation and the mechanisms involved.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Mandel ID, Gaffar A. Calculus revisited. A review. J Clin Periodontol 1986;13:249-57.
2. Roberts-Harry EA, Clerehugh V. Subgingival calculus: Where are we now? A comparative review. J Dent 2000;28:93-102.
3. White DJ. Dental calculus: Recent insights into occurrence, formation, prevention, removal and oral health effects of supragingival and subgingival deposits. Eur J Oral Sci 1997;105(5 Pt 2):508-22.
4. Tan B, Gillam DG, Mordan NJ, Galgut PN. A preliminary investigation into the ultrastructure of dental calculus and associated bacteria. J Clin Periodontol 2004;31:364-9.
5. Newman HN. The development of dental plaque. In: Harris NO, Christen AG, editors. Primary Preventive Dentistry. East Norwalk, CT: Appleton & Lange; 1994. p. 19-58.
6. Hinrichs JE. The role of dental calculus and other predisposing factors. In: Newman MG, Takei H, Klokkevold P, Caranza FA, editors. Carranza's Clinical Periodontology. St. Louis MO: WB Saunders; 2006. p. 170-92.

7. Moorer WR, Ten Cate JM, Buijs JF. Calcification of a cariogenic *Streptococcus* and of *Corynebacterium* (*Bacterionema*) *matruchotii*. J Dent Res 1993;72:1021-6.

8. Baris O. Isolation, Characterization and Identification of the Bacteria that are Effective in Dripstone Formation in the Caves in Erzurum. Atatürk University, Graduate School of Natural and Applied Sciences Department of Biology, PhD Thesis, Erzurum/TURKEY; 2009. p. 135.

9. Boquet E, Boronat A, Ramos-Cormenzana A. Production of calcite (Calcium Carbonate) crystals by soil bacteria is a general phenomenon. Nature 1973;246:527-9.

10. Demir T, Baris O, Zor E. Investigation of *in vitro* mineral forming bacterial ısolates from subgingival calculus. Arch Clin Exp Surg 2014;3:153-60.

11. Marvasi M, Gallagher KL, Martinez LC, Pagan WC, Santiago RE, Vega GC, *et al*. Importance of B4 medium in determining organomineralization potential of bacterial environmental isolates. Geomicrobiol J 2012;29:916-24.

12. Wilson K. Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol 2001; [Chapter 2:Unit 2.4].

13. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403-10.

14. Jin Y, Yip HK. Supragingival calculus: Formation and control. Crit Rev Oral Biol Med 2002;13:426-41.

15. Dahlén G, Konradsson K, Eriksson S, Teanpaisan R, Piwat S, Carlén A. A microbiological study in relation to the presence of caries and calculus. Acta Odontol Scand 2010;68:199-206.

16. Ennever J. Intracellular calcification by oral filamentous microorganisms. J Periodontol 1960;31:304-7.

17. Vogel JJ, Ennever J. The role of a lipoprotein in the intracellular hydroxyapatite formation in *Bacterionema matruchotii*. Clin Orthop Relat Res 1971;78:218-22.

18. Tan BT, Mordan NJ, Embleton J, Pratten J, Galgut PN. Study of bacterial viability within human supragingival dental calculus.

19. Friskopp J. Ultrastructure of nondecalcified supragingival and subgingival calculus. J Periodontol 1983;54:542-50.

20. Zbinden A, Mueller NJ, Tarr PE, Eich G, Schulthess B, Bahlmann AS, *et al. Streptococcus tigurinus*, a novel member of the *Streptococcus mitis* group, causes invasive infections. J Clin Microbiol 2012;50:2969-73.

21. Kuklinska D, Kilian M. Relative proportions of *Haemophilus* species in the throat of healthy children and adults. Eur J Clin Microbiol 1984;3:249-52.

22. Petsios A, Nakou M, Manti F. Microflora in adult periodontitis. J Periodontal Res 1995;30:325-31.

23. Liljemark WF, Bloomquist CG, Uhl LA, Schaffer EM, Wolff LF, Pihlstrom BL, *et al*. Distribution of oral *Haemophilus* species in dental plaque from a large adult population. Infect Immun 1984;46:778-86.

24. Moolya NN, Thakur S, Ravindra S, Setty SB, Kulkarni R, Hallikeri K. Viability of bacteria in dental calculus – A microbiological study. J Indian Soc Periodontol 2010;14:222-6.

25. Long PA, Sly LI, Pham AV, Davis GH. Characterization of *Morococcus cerebrosus* gen. nov., sp. nov. and comparison with *Neisseria mucosa*. Int J Syst Bacteriol 1981;31:294-301.

26. Huse SM, Ye Y, Zhou Y, Fodor AA. A core human microbiome as viewed through 16S rRNA sequence clusters. PLoS One 2012;7:e34242.

27. Smith KS, Ferry JG. Prokaryotic carbonic anhydrases. FEMS Microbiol Rev 2000;24:335-66.

28. Forkman A. The location of carbonic anhydrase in a strain of *Neisseria flava*. Acta Pathol Microbiol Scand B Microbiol Immunol 1972;80:460-6.

29. He XS, Shi WY. Oral microbiology: Past, present and future. Int J Oral Sci 2009;1:47-58.

30. Chahal N, Rajor A, Siddique R. Calcium carbonate precipitation by different bacterial strains. Afr J Biotechnol 2011;10:8359-72.