Study of Bacterial Viability within Human Supragingival Dental Calculus

Benjamin T.K. Tan,* Nicola J. Mordan,† Jason Embleton,‡ Jonathan Pratten,‡ and Peter N. Galgut*

Background: There is evidence that supragingival calculus contains unmineralized channels and lacunae. The purpose of this study was to investigate the viability of bacteria within these areas.

Methods: Supragingival calculus harvested from patients with moderate to severe chronic periodontitis was immediately frozen to –70°C. Six samples were cryosectioned, stained with a bacterial viability kit, and examined with fluorescence microscopy. Controls comprised heat treatment of cryosections prior to staining. Four additional samples were stained and examined whole in a confocal laser scanning microscope (CLSM). Nine additional samples were prepared for bacterial culture, after initial irradiation with ultraviolet light to kill viable organisms on the covering plaque layer. Test samples were crushed to expose internal bacteria, while two controls were used without crushing.

Results: Viable bacteria, as identified using the bacterial viability stain, were found within cavities/lacunae in supragingival calculus cryosections. Similar results were obtained from whole calculus samples using CLSM. Of the nine experimental samples where bacterial culture was attempted, five provided positive bacterial culture under both aerobic and anaerobic conditions; one showed positive growth under aerobic conditions only; while one showed no bacterial growth. The controls showed no bacterial growth.

Conclusions: From this study, it appears that viable aerobic and anaerobic bacteria may be present within supragingival calculus, specifically within the internal channels and lacunae. Clinically, this may be important, since incomplete removal of supragingival calculus may expose these reservoirs of possible pathogenic bacteria and be a factor in the recurrence of periodontal diseases after treatment. *J Periodontol 2004;75:23-29.*

KEY WORDS

Bacteria, aerobic/growth; bacteria, anaerobic/growth; dental calculus, supragingival.

* Department of Periodontology, Eastman Dental Institute for Oral Health Care Sciences, University College London, U.K.
† Department of Biomaterial Sciences, Eastman Dental Institute for Oral Health Care Sciences.
‡ Department of Microbiology, Eastman Dental Institute for Oral Health Care Sciences.

Evidence for the role of calculus in the initiation and progression of periodontal diseases is inconclusive. While it is accepted that calculus in itself is not harmful, its presence in association with dental plaque may influence the severity and progression of periodontal diseases. It is thought that the role of calculus is associated principally with its physical character, in that it is plaque retentive and may impede natural and mechanical oral hygiene activities. However, there is evidence that calculus is not a solid mineralized mass but has a porous, spongy nature as established histologically,[1] and in scanning electron microscopy (SEM)[2,3] and transmission electron microscopy (TEM)[4,5] studies. It has been suggested that dental calculus may act as a reservoir for irritating substances such as endotoxins and bacterial antibodies.[6-8] Such a model might explain the conflicting results from some epidemiological and clinical studies with regards to calculus and periodontal diseases. In particular, Bergström[9] found a positive association, independent of plaque, between supragingival calculus and the gingival index in a population with a high standard of dental awareness and suggested that in individuals with low plaque levels, supragingival calculus might contribute to gingival inflammation. In addition, supragingival calculus has been implicated in gingival inflammation[10] and was positively associated with gingival recession.[11]

It has been estimated that 20% of dental calculus is made up of organic matrix.[12] Non-mineralized cavities, containing microorganisms and extracellular substance

resembling that of the covering soft plaque, have been found within the body of supragingival calculus.[4,5] It has been demonstrated that the environment within a biofilm is able to support viable bacterial communities through molecular diffusion of nutrients through channels,[13] and that calculus can become permeated by substances such as endotoxins within 24 hours.[6] The possibility therefore exists that a sustainable environment for oral bacteria may be found within the dental calculus and that such organisms may remain viable for some time. Bacteria have been cultured successfully from samples of supragingival and subgingival calculus,[14] though the superficial covering of plaque was included with the calculus samples in the study.

A well-established technique for demonstrating bacterial viability comprises fluorescent stains that differentiate live and dead microorganisms. This technique has been used to demonstrate the viability of oral microorganisms in numerous biofilm studies,[15-18] and the stains have been shown to penetrate throughout the biofilm.[18] The aim of the present study was to investigate the viability of bacteria within the unmineralized channels and lacunae traversing supragingival calculus using this viability stain and culturing techniques.

MATERIALS AND METHODS

Six samples of supragingival calculus were removed from patients presenting with moderate to advanced chronic periodontitis as part of their periodontal treatment at the Department of Periodontology, Eastman Dental Institute for Oral Health Care Sciences. Patients from whom samples were harvested had active periodontal disease with substantial calculus accumulation. Additional criteria for acceptance into the study were: 1) no antimicrobial therapy; 2) no professional prophylaxis for at least 6 months prior to harvesting the samples; and 3) medically healthy status. Care was taken to obtain large, single pieces and to maintain the cross-sectional integrity of the calculus samples. Immediately upon removal, the harvested calculus samples were placed, without fixative, in a –70°C freezer.

Fluorescence Microscopy

The supragingival calculus samples were removed from the freezer and placed onto a thin layer of embedding compound (provided by Raymond A. Lamb, Queen Mary College, University of London, London, U.K.) on the surface of a cryostat chuck. Additional compound was then placed over the top of the calculus so that the entire sample was embedded, then returned to a –70°C freezer for 20 minutes. Sections 20 µm thick were cut on a cryostat[§] at –19°C and placed on clean microscope slides. Only sections from the middle portion of the calculus sample were selected for investigation, in an attempt to avoid confusion with the external plaque. Negative controls comprised a section from each sample, which was placed above a Bunsen flame for 1 minute to kill any bacteria present prior to staining.

The fluorescence stain used was a bacterial viability kit.[‖] The kit comprises two nucleic acid stains, SYTO 9 and propidium iodide. SYTO 9 green fluorescent stain penetrates and stains most bacteria in a population, both those with intact membranes and those with damaged membranes. Propidium iodide red fluorescent stain enters only dead bacteria with compromised membranes. Thus, the differential staining obtained is a result of only the green SYTO 9 entering intact bacteria; when both stains enter damaged cells, the red propidium iodide overwhelms the SYTO 9. The stains were supplied in two dye solutions: the first contained 1.67 mM SYTO 9 and 18.3 mM propidium iodide in dimethyl sulphoxide (DMSO) and the second, 1.67 mM SYTO 9 and 1.67 mM propidium iodide in DMSO. Volumes of each stain (3 µl) were transferred to 10 ml of artificial saliva salt medium consisting of 0.83 g NaCl, 0.2 g $CaCl_2$, and 0.2 g KCl per liter.[15] Stain suspension (15 µl) was added to each section of calculus. The samples were then incubated at room temperature in the dark for 15 minutes.

The stained specimens were observed by epifluorescence microscopy using a microscope fitted with a mercury-arc lamp.[¶] The objective lens was an ultra-long working distance (ulwd) (×40).[#]

A color video camera[**] was attached to the microscope. The camera output was connected to a personal computer fitted with a composite board.[††] With this board, individual frames were captured from the video camera.

Confocal Laser Scanning Microscopy

An additional four calculus samples were stained as described above but examined whole in a CLSM. One of the principal properties of the CLSM is its ability to image a virtual section through the middle of a specimen without recourse to sectioning. It was used to image cavities within the calculus while avoiding the possibility that external soft plaque may have been physically introduced.

The specimens were observed under a microscope, with a UV Krypton laser.[‡‡] The objective lens was a 40× UV lens. The images were captured directly into a host computer and recorded onto a compact disc.

Bacterial Culture

Nine additional samples of supragingival calculus were used for this part of the study. In order to kill viable

§ Bright Instrument Company Ltd., Huntingdon, U.K.
‖ *Bac*Light LIVE/DEAD, Molecular Probes Inc., Eugene, OR.
¶ Carl Zeiss, Oberkochen, Germany.
# CDPlan 40, Olympus, Southall, Middlesex, U.K.
** JVC TK-C1381, BRSL, Newbury, U.K.
†† Mutech Image/VGA, Optimum Vision Ltd., Hampshire, U.K.
‡‡ Leica TCS SP CLSM, Leica, Cambridge, U.K.

organisms present on the plaque layer covering the supragingival calculus samples, these samples were first placed under ultraviolet light overnight. The samples were shaken on a vibrator§§ to enable all surfaces of the calculus to be exposed to the UV light. The samples were then weighed.

Two control samples were suspended whole in phosphate buffered saline‖‖ and vortexed for 10 seconds. In the study group, each calculus sample was crushed with a mortar and pestle and suspended in 10 ml of PBS. Suspensions were centrifuged at 5,000 rpm for 10 minutes,¶¶ the pellet resuspended in 1 ml of PBS, and centrifuged at 10,000 rpm for 10 minutes.## This pellet was resuspended in 800 µl of PBS prior to culturing.

The total aerobic counts were performed on Columbia blood agar base (CBA)*** and anaerobic counts on Fastidious anaerobe agar (FAA),††† each containing 5% defibrinated horse blood.‡‡‡ For aerobic counts, the plates were incubated in atmospheric air for 24 hours, while for anaerobic counts, incubation was for 96 hours at 37°C in 10% H_2, 10% CO_2, and 80% N_2.

RESULTS

Fluorescence Microscopy

After application of the bacterial viability stain, viable bacteria fluoresced green, while dead bacteria appeared red. In sections viewed under unfiltered fluorescent light, these were seen as bright green/yellow and red/orange colors, respectively. Autofluorescence due to the high calcium phosphate content of the calculus appeared as dark green to orange.

No viable bacteria were observed in the heat-killed control sections, which had an overall mottled orange color under fluorescent light. Viable bacteria, which appeared as small dots or areas of bright green fluorescence, were frequently detected within the calculus in the experimental sections. In all samples, these viable bacteria were noted lining the walls of apparent non-mineralized cavities, which appeared as dark areas within the calculus (Fig. 1). Viable bacteria were also observed lining non-mineralized channels in the calculus leading from the lacunae (Fig. 2). A group of red, non-viable organisms were noted at the edge of this lacuna, and demonstrated how the red fluorescence was not as readily resolved as the green with this technique. Groups of viable and dead bacteria sometimes appeared to be present within mineralized areas of the calculus (Fig. 3). However, due to the thickness of the sections, it was probable that these fluorescent organisms were also within non-mineralized areas as indicated by the dashed lines (Fig. 3). All six samples of supragingival calculus examined in this manner demonstrated the presence of viable and non-viable bacteria, though only three of the samples provided large enough groups to readily demonstrate the location.

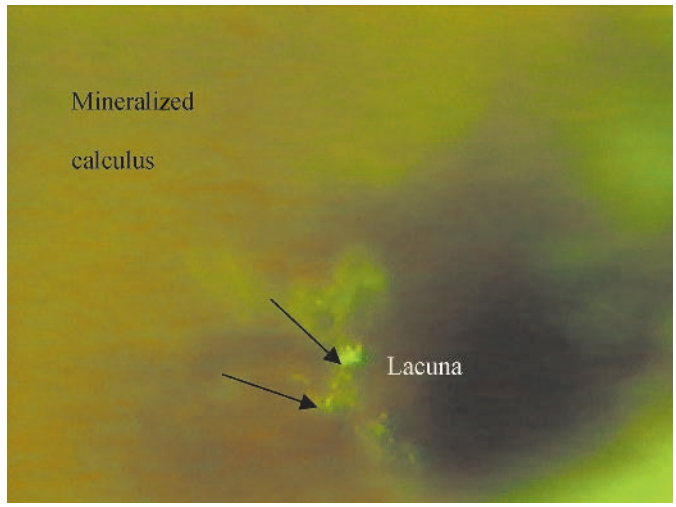

Figure 1.
Unfiltered fluorescence image of mature supragingival calculus after application of the bacterial viability stain. Apparently viable bacteria (arrows) were observed on the periphery of a small lacuna within the calculus. The lacuna appeared dark, and the mineralized calculus was green/orange. (Original magnification ×400.)

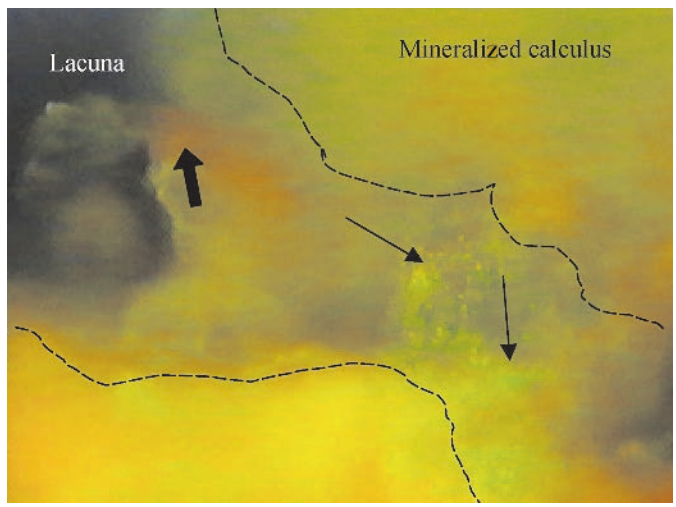

Figure 2.
Unfiltered fluorescence image showing viable bacteria (thin arrows) in the middle of a channel (dashed black line). An area of dead cells can also be seen (large arrow). (Original magnification ×400.)

Confocal Laser Scanning Microscopy

In all four samples stained for CLSM, the stain was judged to have penetrated the entire sample since flu-

§§ Vortexgenie 2, Scientific Industries, Bohemia, NY.
‖‖ Oxoid, Hampshire, U.K.
¶¶ Eppendorf 5804R, Hamburg, Germany.
Eppendorf 5415D.
*** Oxoid, Basingstoke, U.K.
††† Bioconnections, Leeds, U.K.
‡‡‡ E and O Laboratories, Bonnybridge, U.K.

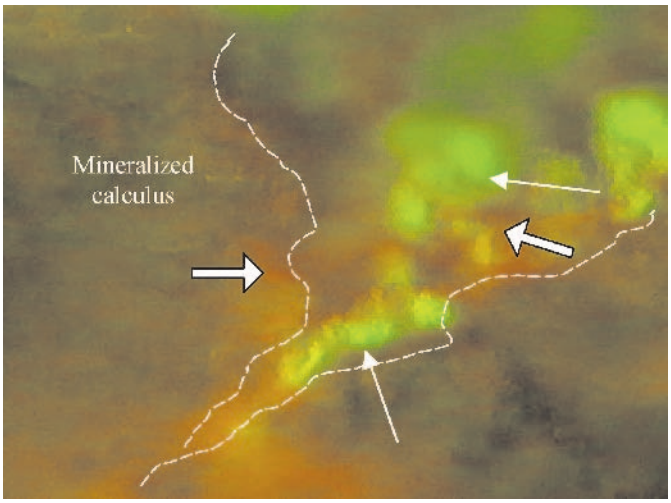

Figure 3.
Unfiltered fluorescence image with some groups of viable (thin arrows) and dead (large arrows) bacteria within the calculus. These groups are within a lacuna (dashed white line), which is more difficult to discern due to the plane of this section. (Original magnification ×400.)

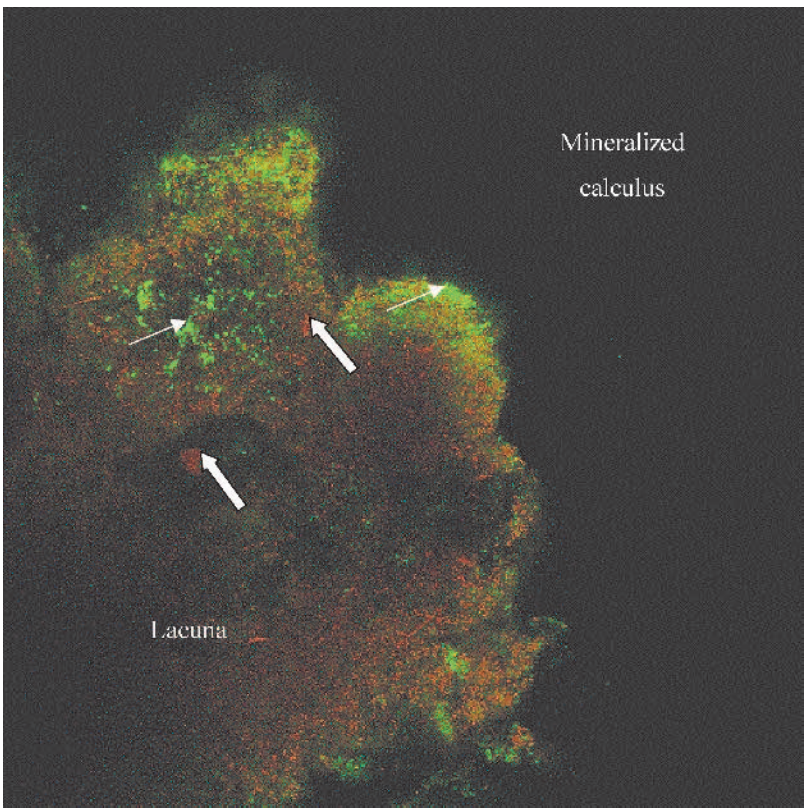

Figure 4.
A CLSM image through a lacuna within an intact piece of supragingival calculus after staining with the bacterial viability stain. The auto-fluorescence due to the mineral has been filtered out so that it appears dark. Live (thin arrows) and dead (large arrows) bacteria can be seen, which appear to traverse the whole of the lacuna. (Original magnification ×40.)

orescent organisms were observed throughout the calculus. The resolution of the CLSM was such that individual organisms could be visualized and, in particular, the red-staining bacteria could be resolved. The CLSM software filtered out auto-fluorescence due to the mineral content of calculus, and those mineral areas appear dark with this technique. It should be noted that these dark areas do not represent lack of staining.

Within these whole calculus samples, many small dots of green fluorescence were observed, sometimes grouped, which probably represent individual microorganisms and small colonies of viable bacteria, respectively (Fig. 4). Present among these viable green fluorescent bacteria were also red fluorescent microorganisms, which signified non-viable bacteria (Fig. 4). These individual microorganisms or groups of bacteria were located in the cavities of the unsectioned specimens and, due to the optical sectioning of the CLSM, these were estimated to be well within the calculus. With this technique, stained microorganisms were observed throughout the lacunae.

Figure 5 is a montage of a series of sections through a large non-mineralized area (to the left of the images) within a whole calculus sample. The optical sections were 1 µm, but for this montage, only alternate sections are shown. Although microorganisms in this lacuna were more often located on its internal edge, where both live and dead cells were observed, bacteria were observed throughout the lacuna.

Bacterial Culture

The results of total number counts for bacterial colonies and possible types of bacteria cultured from supragingival samples are detailed in Table 1. The control samples (C1 and C2) showed no growth on the plates incubated in both aerobic and anaerobic conditions. One study specimen (1) also showed no bacterial growth in either aerobic or anaerobic conditions. One sample (2) showed positive bacterial culture, 32 colonies of two types of bacteria, under aerobic conditions only. The other five samples showed positive bacterial culture under both aerobic and anaerobic conditions. Sample 7 produced the highest counts, with 438 colonies of three types under aerobic conditions and 1,054 colonies of four types under anaerobic conditions.

DISCUSSION

This investigation sought to confirm the presence of viable bacteria within the non-mineralized cavities found in supragingival calculus using an immunofluorescent viability stain and culturing methods. Earlier TEM

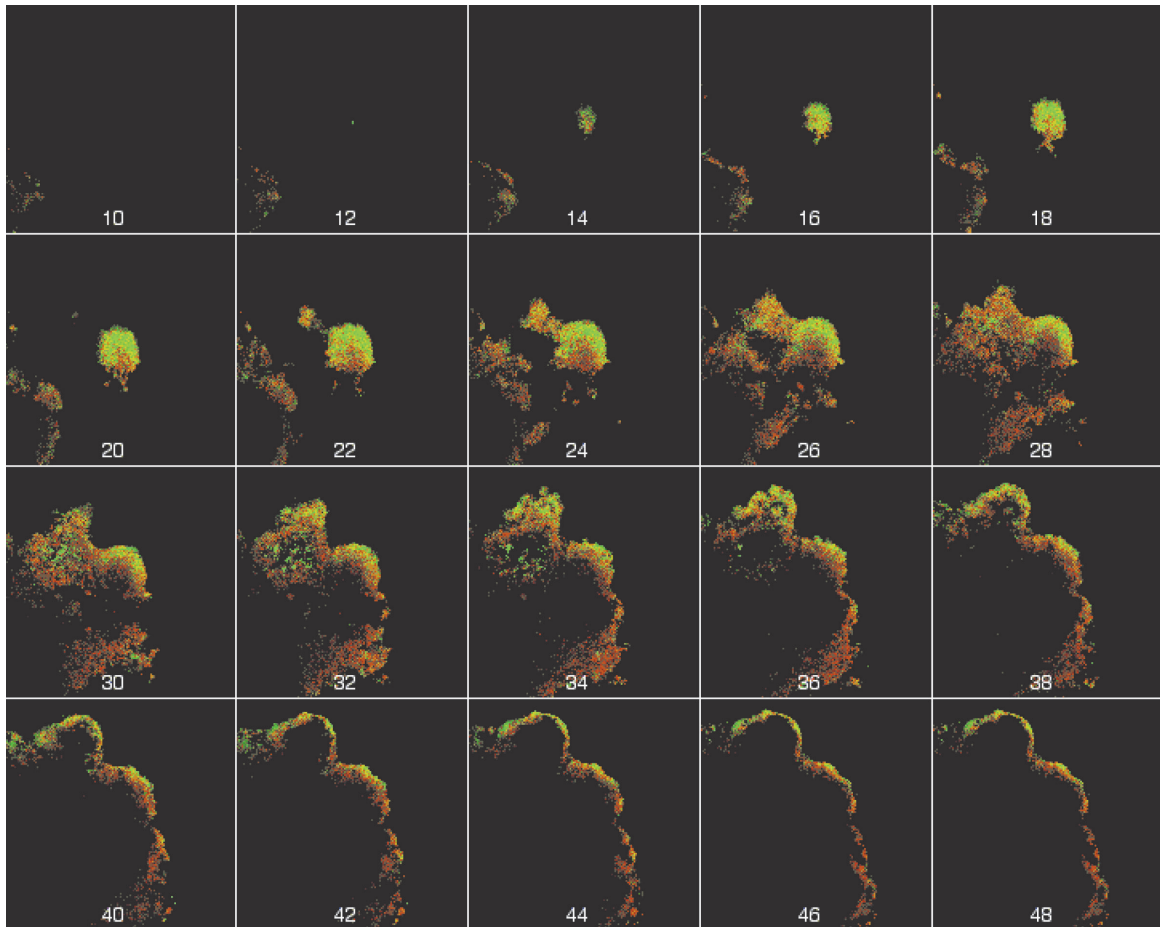

Figure 5.
Montage of a series of CLSM images taken from a channel traversing calculus, taken at 2 µm intervals. Images 10 and 12 show the region immediately preceding the start of the channel. A group of viable bacteria (images 14-28) and smaller viable groups together with areas of dead organisms (images 26-38) were observed filling these lacunae. Toward the opening of the channel to the oral cavity (images 38-48), colonies of both viable and non-viable organisms were present, but these were greatly reduced and confined to the periphery of the lacunae only.

studies have indicated that some of the bacteria within the non-mineralized channels and islands in supragingival calculus remain intact and appear viable.[4,5] Although there are reports in the literature implying that calculus may be essentially mineralized "dead" organic material,[19,20] it has been shown that calcification can occur in a culture of live bacterial colonies.[21] It is therefore possible that some microorganisms may readily calcify while others may not, leading to the creation of pockets of non-mineralized bacteria within the otherwise calcified body.

The bacterial viability stain kit has been used in previous studies[15-18] and is an established technique used to distinguish between viable and non-viable bacterial cells in biofilms. Our report represents the first account of the presence of viable bacteria within calculus using this investigative technique. The kit equates the presence of intact plasma membranes with bacterial viability. False-positive and -negative results can occur, for example, after exposure to agents that render bacteria non-viable but do not compromise the integrity of the plasma membrane, such as formaldehyde, and any treatment that may temporarily compromise the bacterial cell membrane. This did not present a problem in this study, because the specimens were not subjected to any chemical fixatives or other processing. However, indicators of metabolic activity or membrane potential may be useful to further assess bacterial viability.

The bacterial viability stain kit was used initially on cryosectioned material and subsequently on whole calculus samples, in order to overcome the possibility of bacteria being introduced into the calculus samples from the covering plaque during cryosectioning. Since both of these techniques provided similar results, it is highly unlikely that the viable organisms identified in this study were present due to contamination or processing errors. These fluorescence-based stain investigations have indicated that some of the bacteria within the channels and non-mineralized lacunae in supragingival calculus

Table 1.

Total Number of Bacterial Colonies and Possible Types of Bacteria Cultured from Supragingival Samples

		Aerobes		Anaerobes	
Calculus Sample	Mass (g)	Total Colonies	Total Types of Bacteria	Total Colonies	Total Types of Bacteria
C1	0.007	0	0	0	0
C2	0.0017	0	0	0	0
1	0.0058	0	0	0	0
2	0.0054	32	2	0	0
3	0.0003	6	2	2	1
4	0.0024	8	2	322	4
5	0.0261	6	3	20	3
6	0.0333	52	2	86	3
7	0.0033	438	3	1,054	4

appeared viable, and verify the findings from our ultrastructural TEM studies that indicated apparently viable, intact bacteria.

These findings may be of clinical importance, as bacteria within the calculus may act as a reservoir of organisms that might play a crucial role in the etiology and resolution of infective conditions of the gingivae and periodontium. If the bacteria in calculus are vital, they may well be releasing toxic metabolic by-products that may leach from the calculus, initiating inflammatory responses in the oral soft tissues. If the bacteria are nonvital, the by-products from their degradation, such as the lipopolysaccharide cell membrane remnants, might be leached from the calculus into the tissues. This has led Mandel and Gaffar[22] to describe calculus as a "toxic waste dump site" and a slow-release device releasing toxic and pathogenic products into the adjacent tissues. Therefore, it is conceivable that dental calculus is not the benign substance that it is currently considered to be.

The viability of bacteria within the supragingival calculus samples was also examined by bacterial culture. Sidaway[19] reported successful bacterial culture from samples of supragingival and subgingival calculus, although the superficial covering of plaque was probably included in the cultures. In the present study, we attempted to eliminate contamination due to the overlying plaque by first placing the supragingival calculus samples overnight under ultraviolet light on a shaker to enable all surfaces of the calculus to be exposed to the UV light. This method appeared to be successful since both control specimens showed negative aerobic and anaerobic bacterial culture. Although specific periopathogenic organisms were not identified in this study, the aerobic species grown in five out of seven samples indicate the possibility for the presence of pathogens within supragingival calculus. The identification of these organisms is the subject of a further study.

In this study, we examined 17 samples of supragingival calculus by one of three different methodologies, and 16 of the specimens provided strong evidence indicating that microorganisms contained within the calculus, probably within the lacunae and channels, were viable. Furthermore, results of the culture data imply that some of these bacteria are capable of growth when placed in a suitable environment, as might be the scenario with incomplete removal of calculus.

Several clinical studies have examined the effects of personal oral hygiene versus mechanical debridement and have shown that it is only with thorough removal of the mineralized calculus that improvements in periodontal indices are attained.[23,24] Also, individuals with low plaque levels show a positive association, independent of plaque, between supragingival calculus and gingival index, suggesting that supragingival calculus might contribute to gingival inflammation.[8] The presence of viable bacteria within supragingival calculus may explain some of the conflicting results of the epidemiological and clinical studies and reinforce the case for ensuring complete removal of these deposits from the tooth surface.

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Correspondence: Dr. Peter N. Galgut, 28 Rundell Crescent, London NW4 3BP, U.K. E-mail: peter@periodontal.co.uk.

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