Calcification of Selected Strains of *Streptococcus mutans* and *Streptococcus sanguis*

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Nine strains of cariogenic *Streptococcus mutans* and two strains of *Streptococcus sanguis* were tested for their ability to form hydroxyapatite. The cells were examined by X-ray diffraction and electron microscopy for apatite crystals after growth in a synthetic calcification medium. Each of the test isolates, except for one strain of *S. sanguis*, produced intracellular mineral. Two strains of *S. mutans* formed both intra- and extracellular crystals. There was no apparent relationship between calcifiability and serotype.

Calcium phosphate similar to hydroxyapatite, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, is the major mineral of vertebrate hard tissues. It is also identified as a constituent of the mineralized deposit of calcified oral microorganisms which develop in dental calculus at the tooth gingival interface (3). The present study was undertaken to determine the calcification potential of selected oral streptococci which are known to colonize on tooth surfaces and have been implicated in dental caries (18).

Calcification from pure cultures was first reported for *Bacterionema matruchotii*. This filamentous member of the oral microflora of man and some lower animals forms intracellular apatite (4, 19). A recent survey of 14 oral isolates obtained from humans and marmosets showed that four enteric species also form apatite. Calcification on a pure culture basis, therefore, is not unique to *B. matruchotii*, but neither is it universal among oral microorganisms. One of the noncalcifying isolates in the survey was identified as *Streptococcus salivarius* (8). Subsequently a streptococcal variant obtained from *B. matruchotii* calcified, forming intra- and extracellular deposits (6). These findings indicated that streptococci of recognized lineage should be studied, especially those associated with oral disease. Nine strains of cariogenic *S. mutans* representative of the established serotypes (1) and two strains of *S. sanguis* were examined for calcification.

**MATERIALS AND METHODS**

**Strains.** Nine strains of *S. mutans* including serotypes a, b, c, d, and E, and two strains of *S. sanguis*, Types I and II, were examined for calcification. *S. salivarius* 125C served as a noncalcifying control. The strain designation and serotypes are presented in Table 1.

**Media.** Each organism was grown at 37°C in 250 ml of the synthetic medium of Ennever, Vogel, and Streckfuss (10) for 14 days. The pH of the medium was determined before and after incubation. The cultures were harvested by centrifugation at 27,000 × g for 5 min at 4°C and washed three times with deionized water. The concentrated cells of each isolate were then frozen at -15°C and divided into two equal parts. One portion was air-dried at 45°C and radio-frequency ashed for 2 to 4 h at 55°C for X-ray diffraction. The remaining portion of each sample was processed for electron microscopy as previously reported (10).

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<th>Table 1. Calcification of streptococcal strains</th>
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<tr>
<td><strong>Strain</strong></td>
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<td><em>S. mutans</em></td>
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<tr>
<td>AHT-12</td>
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<tr>
<td>FA-1</td>
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<td>GS5</td>
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<td>PK1</td>
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<td>SL-1</td>
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<td>6715</td>
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<td><em>S. sanguis</em></td>
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<tr>
<td>Type II</td>
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<td><em>S. salivarius</em></td>
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+a Intra- and extracellular.
+b Intracellular.
+c *S. mutans* with antigen reacting with Lance field group E (2, 18).
RESULTS

Growth. The initial pH of the medium was 7.2 and after incubation was 6.9. S. mutans OMZ-61, AHT-12, GS5, PK1, S3, SL-1, and 6715 produced rapid growth reaching a maximum after 30 h of incubation. The remaining isolates of S. mutans (FA-1 and LM7), S. sanguis Types I and II, and S. salivarius 125 C grew sparsely. The slow-growing isolates were subsequently inoculated into triplicate volumes of medium to obtain a sufficient mass of cells for X-ray diffraction.

X-ray diffraction. An X-ray diffraction pattern of dental calculus is presented for reference in Fig. 1A. The test isolates produced patterns for apatite except S. mutans FA-1 and S. sanguis Type II (Fig. 1G and H). Figure H also represents the noncalcifiable control, S. salivarius 125C. Patterns representative of the serotypes of S. mutans and S. sanguis Type I are illustrated in Fig. 1B through F.

The diffuse bands in the diffraction patterns indicate the apatite crystals were small. With S. mutans strains GS5 and LM7, (Fig. 1C and E), the pattern was faint because the concentration of apatite in the ashed material was just at the point of X-ray resolution. Apatite with S. mutans strain FA-1 was not detectable by X-ray diffraction (Fig. 1G) but crystals typical of apatite were found by electron microscopy (Fig. 2B).

Electron microscopy. Characteristic needle-like intracellular crystals of apatite produced by one isolate of each serotype of S. mutans and for S. sanguis Type I are illustrated in Fig. 2A through G. Figures 2H and I show the lack of crystals respectively for the noncalcifying S. sanguis Type II and S. salivarius 125C. Extracellular crystallinity was noted in S. mutans OMZ-61 (Fig. 2A) and S3 (Fig. 2D). Calcification in S. mutans strain GS5 (Fig. 2C) and SL-1 (Fig. 2E) appeared to be associated with the cell membrane (arrows) by the darker electron dense areas at the periphery of the cells.

DISCUSSION

A variety of genera of oral microorganisms are known to calcify when implanted in the rat peritoneum (17). The defined cultural system utilized in this study, however, provided a means to observe relative differences in calcifiability with pure cultures. Biological apatite was readily detectable in the rapid-growing isolates, whereas the slower developing strains produced weakly detectable mineral or were noncalcifiable. These observations and the presence of extracellular crystallinity indicate the need to relate crystal nucleation to the growth rate of the streptococci. Once crystal nucleation occurs, crystal growth is favored in the medium. Apatite was detectable after 5 days of incubation and from 12 to 14 days with the rapid- and slow-growing strains, respectively. Under these conditions, it is estimated that about 5% crystallinity is detectable in ashed cell residues by X-ray diffraction. The level of apatite detection raised the question of whether the mass of cells ashed in the noncalcifiable strains was adequate for detection of low levels of crystallinity. S. sanguis Type II and S. salivarius 125C have not produced crystallinity that was detectable by X-ray diffraction or electron microscopy. These isolates, therefore, were repeatedly examined to insure that the mass of cells ashed was in excess when compared with that of the calcifiable forms. No apatite was detectable by X-ray diffraction in the rat strain FA-1, but crystals characteristic of apatite were found by electron microscopy. This probably occurred because mineral could not be sufficiently concentrated in the ashed cell residue.

The variable capacity of isolates from the same species to calcify was demonstrated when S. mutans strain FA-1 was compared with another rat strain, OMZ-61. This isolate in contrast with FA-1 produced rapid growth and extracellular deposits of apatite. A similar observation was made with human strains when LM7 was compared with S3. It was evident that calcifiability was variable with isolates obtained from different and from the same species, and there was no apparent relationship of serotype to calcifiability.

There were no ultrastructural features that provided a basis for the prediction of calcification. Each calcifiable isolate formed intracellular deposits. In S. mutans, GS5 and SL-1, some of the foci appeared oriented at the membrane. In addition, S3 produced extracellular crystals radiating from the cell. This feature may result from frequent breaks along the cell wall where nucleating sites of the membrane are exposed to the medium. The cell-free apatite of OMZ-61 probably represents crystals released from lysed cells. Nucleation of apatite is thought to begin within the cell since extracellular foci were consistently found in the presence of intracellular deposits, as previously reported (6).

The intracellular deposits, some of which appeared associated with cell membranes, suggest that there may be a common nucleating moiety in the streptococci. These observations are also in accord with the proposal that there is
a common nucleator in phosphatic calcifications (4). The mechanism of microbial calcification has been studied using *B. matruchotii* (4, 19, 5) and in this organism, the phospholipid fraction nucleatesapatite (20, 23). Phospholipid is a major component of bacterial membranes and the reduction of these structures in calcifiable cells with a synthetic lathyrogen produces noncalcifiable forms (21). Lipid extracts from vertebrate bone (9), dentin (J. Vogel, personal
Fig. 2. Electron micrographs of isolates. (A) OMZ-61, intra- and extracellular crystals; (B) FA-1, intracellular crystals; (C) GS5, membrane associated (arrow) and intracellular crystals; (D) S3, crystals intracellular and radiating from the cell; (E) SL-1, membrane associated (arrow) and intracellular crystals; (F) LM7, intracellular crystals; (G) S. sanguis Type I, intracellular crystals; (H) noncalcifiable S. sanguis Type II; (I) noncalcifiable S. salivarius 125C; marker bar: 500 nm.
communication), and dental calculus (7) have also nucleatedapatite. A recent report, however, questions the proposal of whether there is a universal calcification factor and suggests instead that calcification is a matter of common mechanism (22).

The potential of the streptococci to produce hydroxyapatite is relevant to expanding studies in microbial calcification. The most significant observation, however, was that organisms known to contribute to the destruction of tooth mineral can themselves form the same mineral in vitro when conditions are favorable.

ACKNOWLEDGMENTS

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LITERATURE CITED