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Calcium Modulates Interactions between Bacteria and Hydroxyapatite

ABSTRACT

Bacterial adhesion onto hydroxyapatite is known to depend on the surface properties of both the biomaterial and the bacterial strain, but less is known about the influence of the composition of the aqueous medium. Here, the adhesion of *Streptococcus mutans* and 3 different *Lactobacilli* on powdered hydroxyapatite was shown to change with Ca^{2+} concentration. The effect depends on the surface properties of each strain. Adhesion of *Lactobacillus fermentum* and *salivarius* (and of *Streptococcus mutans* at low Ca^{2+}) was enhanced with increasing Ca^{2+} concentration. *Lactobacillus casei* was efficiently removed by adhesion on hydroxyapatite, even without Ca^{2+} addition, and the effect of this ion was only marginal. The results are interpreted in terms of Ca^{2+}-mediated adhesion, and relative to the hydrophobic properties of each strain and the electrical properties of the bacterial and solid surfaces (electrophoretic mobility).

KEY WORDS: hydroxyapatite, bacteria, surface properties, interactions.

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INTRODUCTION

Hydroxyapatite (HAP) constitutes more than 90% of dental enamel (de Aza and de Aza, 2004). Upon immersion in oral fluid, synthetic HAP (or any other bare inorganic biomaterial) rapidly develops a proteinaceous film (acquired pellicle), on which bacteria and epithelial cells adhere. All surface properties of the solid are drastically changed by the presence of this film, which defines the net surface charge and the nature of surface chemical groups. Adherent bacteria form a biofilm by adsorption, adhesion, colonization, and invasion of the solid covered by the acquired pellicle (Busscher *et al.*, 1995). Some components of saliva may also promote bacterial aggregation (Weerkamp and McBride, 1980).

This complex picture does not preclude a need for understanding of the behavior predicted by simple models. The Deryaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey and Overbeek, 1948) provides a simple description of the interaction between biomaterials and bacteria by taking into account electrostatic and London dispersion interactions. The former, defined by surface charges, may be attractive or repulsive; the latter, due to correlations between molecular dipoles, are always attractive. Accordingly, van Loosdrecht *et al.* (1987) showed experimentally that both charge and hydrophobicity affect the degree of bacterial adhesion on polymers. The ionic strength and dielectric constant of the medium influence both electrostatic and dispersion interactions; in addition to these generic medium effects (Weerkamp *et al.*, 1988), specific ionic effects by fluoride and acetate (Eifert *et al.*, 1984) have also been described.

This paper reports the results of an experimental study of the interaction of HAP with bacterial cells in simple aqueous solution and in human saliva, focused on the effect of Ca^{2+}. HAP particles, smaller than bacteria, but able to form aggregates of larger size, were used in our effort to understand the interplay between bacterial co-aggregation and hetero-coagulation of bacteria with particles. Electrophoretic mobilities of bacteria and particles provided information on the operating electrical charges. We used hydrophobicity measurements to explore the influence of hydration forces on the interaction. Three oral cavity strains and a more hydrophilic strain, from cheese, were studied. The interaction of particles and bacteria was also observed by optical and electron microscopy. We used the results to explore the role played by Ca^{2+} in the surface phenomena that define bacteria-HAP adhesion.

MATERIALS & METHODS

Powdered hydroxyapatite (Ca/P molar ratio 1.70) was composed of aggregates (*ca.* 10-μm diameter) of well-crystallized nanoparticles (average size, 100 x 10 x 5 nm) with a Brunauer-Emmett-Teller surface area of 23.5 $m^2\ g^{-1}$. Fourier Transform Infrared spectra showed the presence of small quantities of carbonate (weak band at 1460 cm^{-1}). [For further details on the properties of the hydroxyapatite, see García Rodenas *et al.* (2005).]

The strains were *Streptococcus mutans* ATCC25175, *Lactobacillus*

salivarius ATCC11741, and *Lactobacillus fermentum* ATCC14932 from the oral cavity, and *Lactobacillus casei* ATCC393 from cheese. The behavior of hydrophilic bacteria was explored with this latter strain representative of micro-organisms entering the oral cavity with food intake.

All strains were stored at -20°C in skim milk (9% w/v) with yeast extract (0.5% w/v) and subcultivated by 3 successive transfers in LAPTg (Raibaud *et al.*, 1961) broth for *S. mutans* and *L. salivarius*, and MRS broth for *L. fermentum* and *L. casei*. They were grown aerobically in the same media at 37°C for 24 hrs. Active cultures of each strain were centrifuged (5000 *g*, 10 min), and the pellets were suspended in an adequate volume of 0.1% peptone water to yield *ca.* 10^8 CFU mL^{-1}. We determined bacterial counts by plating in the agar medium after incubation at 37°C for 48 hrs.

All other reagents were analytical grade.

We prepared samples for microscopic observations in an optical Karl Zeiss Axilab (immersion objective 100X) by mixing 100 µL bacterial culture (10^8 CFU mL^{-1}) with 200 µL HAP suspension (1.25 g L^{-1}) containing either KNO_3 or $CaCl_2$ (10^{-2} mol L^{-1}), at neutral pH. After 15 min, a 200-µL aliquot was placed on a layer of agar gel for microscopic observation. Ancillary observations were performed in a Philips 515 scanning electron microscope, with de-aggregated (sonicated) HAP samples.

Electrophoretic mobilities were measured in a Rank Brothers apparatus (Cambridge, UK) at various $Ca(NO_3)_2$ concentrations. Constant ionic strength (I = 0.01 mol L^{-1}) was obtained by KNO_3 addition. Before measurement, HAP samples were conditioned for 3 days. Bacterial mobilities were determined in the stationary phase. Measurements were also performed in natural saliva, with and without added Ca^{2+}.

We determined hydrophobicity by measuring the partition between water and toluene, hexadecane, or *p*-xylene (Rosenberg *et al.*, 1980). Bacterial cultures were incubated for 16 hrs, rinsed 3x with physiological solution, and re-suspended until the absorbance at 560 nm was 0.5-0.6. The culture (3 mL) and the organic solvent (1 mL) were mixed and vigorously shaken for 90 sec. After phase separation (15 min), the absorbance of the aqueous fraction was measured. Surface hydrophobicity percentage was calculated as $HB\% = 100\ (1 - A/A_0)$, where A_0 and A are the absorbances before and after extraction.

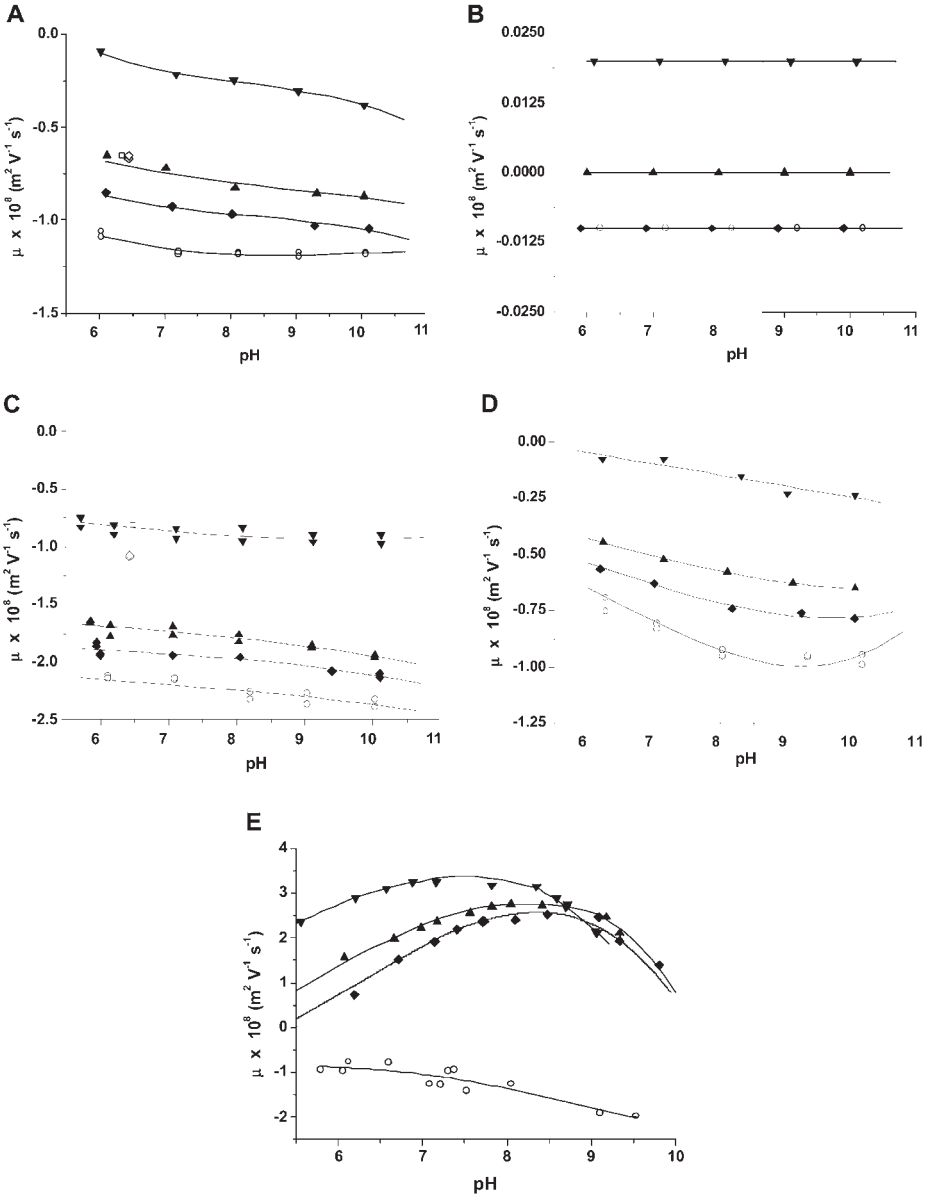

Figure 1. Electrophoretic mobility *vs.* pH profiles of *Streptococcus mutans* **(A)**, *Lactobacillus casei* **(B)**, *Lactobacillus salivarius* **(C)**, *Lactobacillus fermentum* **(D)**, and HAP **(E)** in 0.01 mol L^{-1} KNO_3. $[Ca^{2+}] = 0$ (O), 5×10^{-4} (◆), 1×10^{-3} (▲), and 1×10^{-2} (▼) mol/L. Also shown in (A) and (C), measurements in the presence of natural saliva: $[Ca^{2+}] = 1 \times 10^{-2}$ mol L^{-1} (□); $[Ca^{2+}] = 0$ (◇). Vertical bars indicate SD of triplicate experiments.

For adsorption measurements, HAP was suspended in sterile natural saliva diluted (volume ratio 1:1) with aqueous solutions containing various amounts of $Ca(NO_3)_2$ and KNO_3, with I = 0.02 mol L^{-1} maintained. [Saliva was collected unstimulated from a healthy volunteer individual. No IRB approval of study protocol was necessary.] Saliva was clarified by centrifugation at 20,000 *g* for 20 min at 4°C and sterilized by filtration. Equal volumes of the HAP suspension (10 mg mL^{-1}) and bacterial culture (*ca.* 10^8 CFU) were mixed and left in contact for 2 hrs. Sedimentation of the solid containing HAP and adhered bacteria was achieved by centrifugation at 500 rpm for 1 min. The supernatant with non-adhered bacteria was removed; the solid was re-suspended in 0.1%

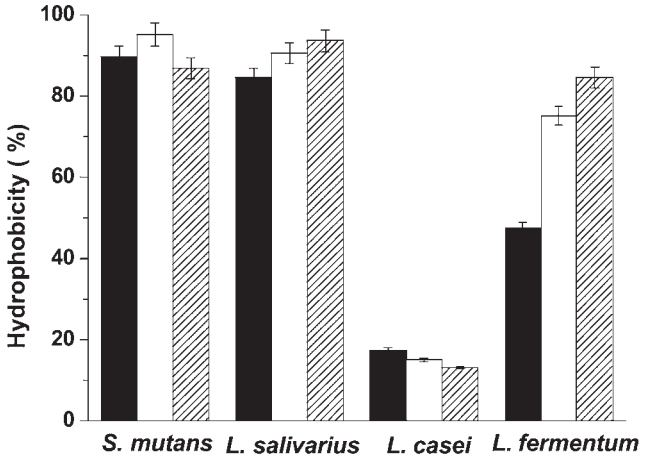

Figure 2. Bacterial hydrophobicities in hexadecane (■), p-xylene (□), and toluene (▨). Bars represent the average of at least 6 measurements, with SD = ± 3%.

peptone water and gently vortex-shaken.

S. mutans and *Lactobacillus* cells adsorbed to the HAP surface were counted by plate count in the agar media; the plates were incubated at 37°C for 48 hrs.

RESULTS

Mobilities of the bacterial strains in aqueous KNO_3 solutions were generally negative in the whole spanned pH and Ca^{2+} concentration ranges (Figs. 1A-1D). The mobility sign (and hence of the surface net charge) was due to carboxylate and other ionized weak acidic groups of the bacterial surface (Cann, 1978; Rose *et al.*, 1993, 1994). The addition of Ca^{2+} made the mobility less negative. Only *S. mutans* showed charge reversal at the highest Ca^{2+} concentration and lowest pH.

Without added calcium, the absolute mobility of *L. casei* was very low, whereas the other strains spanned values in the range (-0.7) - (-2.2) x 10^{-8} (m^2 V^{-1} s^{-1}) at the pH of saliva and I = 0.01 mol L^{-1}. The results for *L. casei* agreed with those reported by Pelletier *et al.* (1997).

S. mutans' mobility in saliva (Fig. 1A) was similar to that observed in 1 x 10^{-3} mol L^{-1} Ca^{2+}, regardless of the Ca^{2+} added to the saliva (0 or 1 x 10^{-2} mol/L). The quenching of the Ca^{2+} influence is attributed to the presence of this ion in the bacterial culture. *L. salivarius*' mobility was not quenched in the same way; the results (Fig. 1C) suggest a Ca^{2+} level in the culture of *ca.* 5 x 10^{-3} mol L^{-1}.

HAP mobility in water was negative in the absence of added Ca^{2+} in the range 5.5 < pH < 9.5. Bell-shaped, positive values were measured in the presence of high Ca^{2+} (Fig. 1E).

L. casei was hydrophilic, whereas *S. mutans* and *L. salivarius* were essentially hydrophobic (Fig. 2). The measurements of *L. fermentum* hydrophobicity in hexadecane deviated considerably from the values in the other 2 solvents. Sweet *et al.* (1987) have reported that hexadecane does not always produce accurate or reproducible results; hence, the abnormally low value measured in hexadecane was discarded. Other effects, such as cell lysis induced by xylene, are not supported by our data.

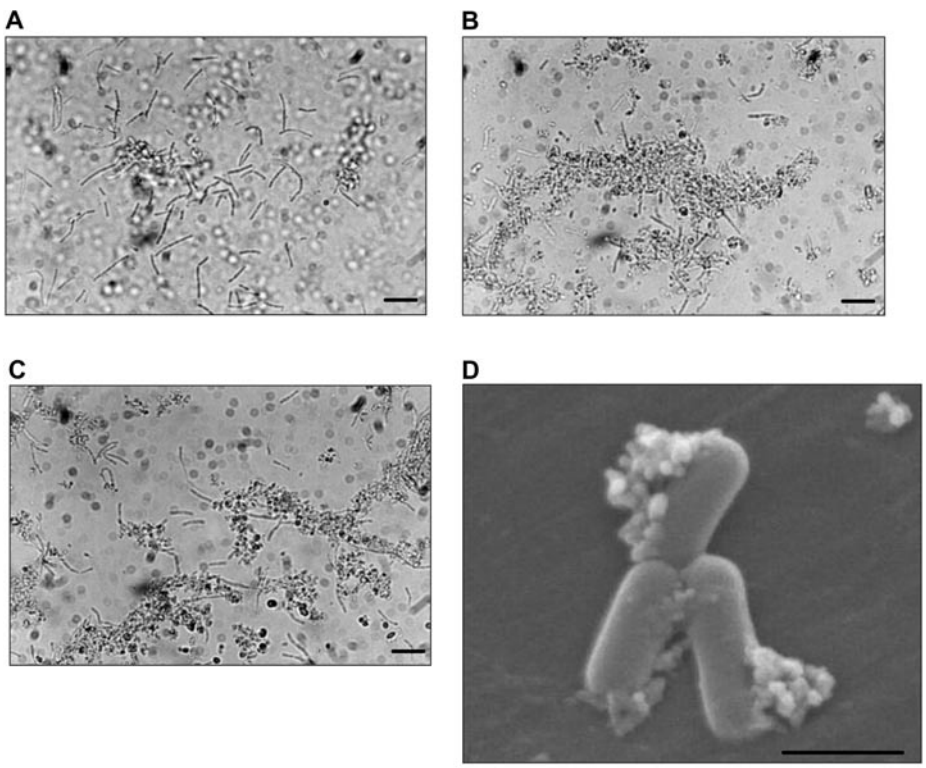

Figure 3. Bacteria-hydroxyapatite interaction. (A-C) Optical photomicrographs (bar = 50 μm) of suspensions containing HAP and *L. salivarius* (A,B) or *L. casei* (C). In **(A)**, no Ca^{2+} was added; in **(B)** and **(C)**, $[Ca^{2+}]$ = 1 x 10^{-2} mol L^{-1}. **(D)** Scanning electron microscope micrograph of suspensions containing *L. salivarius* and hydroxyapatite with $[Ca^{2+}]$ = 1 x 10^{-2} mol L^{-1} (bar = 1 μm).

Microscopic observation of the mixture of cells and HAP in saliva showed that low levels of *L. salivarius* adhesion on HAP were observed when exogenous Ca^{2+} was low, whereas large HAP clusters, including bacteria, were observed at higher-added $[Ca^{2+}]$ (Figs. 3A, 3B). Adsorption was also seen by scanning electron microscopy (SEM) on non-aggregated HAP at high Ca^{2+} (Fig. 3D). *L. casei* adhesion was insensitive to Ca^{2+} addition, being important even in the absence of exogenous Ca^{2+} (Fig. 3C).

The fraction of each strain removed with the HAP precipitate (Fig. 4) depended on the concentration of exogenous Ca^{2+}:

(1) Removal of the 2 highly hydrophobic rods (*L. fermentum* and *L. salivarius*) increased with increasing Ca^{2+} concentration (Fig. 4A).

(2) The same trend, but at high Ca^{2+} concentration, was observed for the hydrophobic coccus *S. mutans* (Fig. 4B).

(3) The highly hydrophilic rod from cheese (*L. casei*) was efficiently

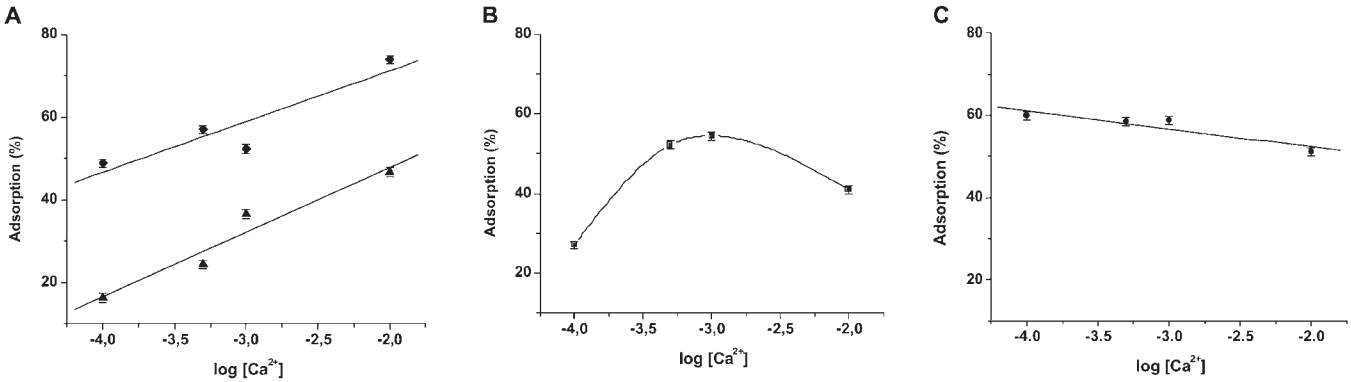

Figure 4. Removal percentage of bacteria by centrifuged hydroxyapatite precipitate (see text) as a function of log [Ca^{2+}]: **(A)** *Lactobacillus salivarius* (▲) and *Lactobacillus fermentum* (◆); **(B)** *Streptococcus mutans;* **(C)** *Lactobacillus casei*. Vertical bars indicate SD of triplicate experiments.

removed even without added Ca^{2+}, and the effect of addition was low or negligible (Fig. 4C).

(4) At high Ca^{2+}, the adsorption of all strains reached values higher than 40%, and the difference between *L. casei* and the other strains largely disappeared.

DISCUSSION

The electrophoretic mobility, μ, is frequently used to calculate the ζ-potential, the electrical potential at the slipping plane. This plane separates the fluid that moves together with the particle from the outer volume in which counter-ions move in the opposite direction; it is usually assumed to coincide with the onset of the diffuse region of the double layer (outer Helmholtz plane, OHP). ζ is determined by the charge density on the particle surface and by the extent of charge neutralization within the OHP. Although, in simple cases, μ is proportional to ζ (Hunter, 1981), in the case of bacteria, their electrical conductivity may affect the relation between μ and ζ (van Loosdrecht *et al.*, 1987). Although Sonohara *et al.* (1995) have derived an equation that calculates ζ potentials from experimental mobility data, with inclusion of the effect of ionic penetrability, it is safer to discuss the charge of the bacteria using raw mobility data (van Loosdrecht *et al.*, 1987).

The mobility/pH profiles for hydroxyapatite in water containing Ca^{2+} have been interpreted in terms of a simple surface complexation model, involving adsorption/desorption of Ca^{2+} and H^+, together with the protolytic equilibria of dissolved phosphate. [Surface complexation models assume that surface charge develops through chemical complexation reactions involving surface species and dissolved ions, and that these interactions can be written as mass-law equilibria. This subject has been extensively treated in the literature; see, *e.g.*, Stumm and Morgan (1996) and Blesa *et al.* (1993). For further details, see García Rodenas *et al.* (2005).] Many factors may influence the mobility of enamel, especially the presence of organic matter; thus, very diverse values of the isoelectric point (pH value at which $\zeta = 0$) have been reported (Kambara *et al.*, 1978).

Bacterial mobility does not change much with pH in the range $6 < pH < 10.5$, because the ionization degree of surface groups is roughly constant. In contrast, Ca^{2+} adsorption affects the mobility appreciably. Positive values in *L. casei* indicate strong chemisorption.

The relation between hydrophobicity and mobility is a matter of debate. Pelletier *et al.* (1997) reported no correlation, whereas van der Mei *et al.* (1988) described a shift in the isoelectric point of a series of *S. salivarius* HB mutant strains that parallels the surface ratio (lipoteichoic)/(teichoic) acids. Both hydrophobicity and mobility measurements are subject to large intrinsic errors, and a detailed analysis of a possible correlation is not warranted. The lower mobility of the hydrophilic strain reported here, however, is beyond doubt.

Hydrophilic interfaces result from high surface density of ionizable groups, and the very low mobilities of *L. casei* must therefore be due to charge neutralization by the strong chemisorption of counter-ions, an effect that may run parallel to ionic conductivity effects (van Loosdrecht *et al.*, 1987). Ca^{2+} is probably already present in high concentrations in the corresponding cultures. The decreased influence of Ca^{2+} addition on the mobility in saliva supports this assertion.

Cann (1978) and Rose *et al.* (1993) have proposed that bacterial adhesion in saliva takes place through irreversible, long-range interactions mediated by polymers, followed by reversible adhesion between anionic groups, mediated by calcium binding. In the oral cavity, tooth enamel covered by the acquired pellicle exposes negatively charged groups to the fluid, and calcium ions provide the natural chemical bridges with the bacterial cells. In filtered sterile saliva, with no acquired pellicle, the surface calcium ions of enamel provide the bridges. In a medium containing HAP particles, bacteria may adhere to the solid and may also co-aggregate. It has been reported that biofilms formed 15 min after inoculation on hydroxyapatite disks consist mainly of single, non-aggregated cells. At longer times, *S. oralis* SK248 (OMZ 607) easily forms spheroidal or ovoid micro-colonies; cylindrical cells, such as those of *A. naeslundii* OMZ 745, also give rise to spheroidal structures on the HAP substrate, with ease (Guggenheim *et al.*, 2001). The present study was carried out under very different conditions, and involved the interaction of bacteria with smaller hydroxyapatite particles aggregated in clusters similar in size to the bacteria. Both co-aggregation and adhesion took place under these conditions, the former leading to larger aggregates at low Ca^{2+} concentrations.

The effect of exogenous Ca^{2+} on adhesion may now be explained. The availability of Ca^{2+}, at low concentrations, determined the affinity of *L. fermentum*, *L. salivarius*, and *S. mutans* for HAP. The behavior of *L. casei* was diverse,

suggesting that a solution layer rich in Ca^{2+} already surrounded this strain. These effects were obvious in spite of the possible Ca^{2+} sequestering effect of saliva components. Adhesion of bacteria and solid particles took place *via* linking Ca^{2+} in all cases; indeed, at the pH of saliva at low Ca^{2+} levels, electrostatic repulsion must operate against simple bacteria-substrate hetero-coagulation. Ca^{2+} not only gives rise to charge reversal, but also provides the bridges to link, through functional groups, HAP and bacterial surfaces. At high Ca^{2+}, bacterial co-aggregation may also be favored, and a decrease in the extent of hetero-coagulation results. This conclusion applies both to *L. casei* and *S. mutans*; this result was not achieved by *L. fermentum* and *L. salivarius*.

The small HAP particles had a marked tendency toward forming large clusters, but the nature of the interaction with bacteria was similar regardless of the size of the cluster. Hetero-coagulation was obvious, but adhesion of small HAP particles onto the larger bacteria was also seen by SEM in sonicated media.

Yoshida *et al.* (1998) described both tightly and loosely adherent bacteria. Although our study did not explore this issue, it is probable that the affinity is not constant, since Ca^{2+} concentration varies, and the solid removes more bacteria at higher Ca^{2+}, including less tightly adherent bacteria.

The interaction between bacterial and HAP surfaces depends not only on the nature and number of available anchoring functional groups, but also on the medium calcium ions that bind the functional groups of the bacteria and the biomaterial. Adhesion would be favored by high calcium concentrations, which in turn may originate from acid attack on HAP at low pH values. Thus, lactic acid formation in the early stages of caries attack on enamel may indirectly generate conditions favorable for further bacterial adhesion. Bacterial co-aggregation may compete with hetero-coagulation for hydrophobic strains at lower solid/bacteria ratios, especially in low Ca^{2+} media.

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REFERENCES

Blesa MA, Morando PJ, Regazzoni AE (1993). Ionic oxides. In: Chemical dissolution of metal oxides. Boca Raton, FL: CRC Press, p. 153.

Busscher HJ, Bos R, van der Mei HC (1995). Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS Microbiol Lett* 128:229-234.

Cann R (1978). Ligand binding in associating systems. *Meth Enzymol* 48:299-307.

de Aza PN, de Aza S (2004). Biocerámicas. In: Biomateriales. Sastre R, de Aza S, San Roman J, editors. Faenza, RA: Editrice Ibérica S.I., p. 54 [article in Spanish].

Eifert R, Rosan B, Golub E (1984). Optimization of an hydroxyapatite adhesion assay for *Streptococcus sanguis*. *Infect Immun* 44:287-291.

García Rodenas L, Palacios JM, Apella MC, Morando PJ, Blesa M (2005). Surface properties of various powdered hydroxyapatites. *J Colloid Interface Sci* 290:145-154.

Guggenheim M, Shapiro S, Gmür R, Guggenheim B (2001). Spatial arrangements and associative behavior of species in an *in vitro* oral biofilm model. *Appl Environ Microbiol* 67:1343-1350.

Hunter RJ (1981). The calculation of zeta potential. In: Zeta potential in colloid science. Ottewill RH, Rowell RL, editors. New York: Academic Press, pp. 59-124.

Kambara M, Asai T, Kumasaki M, Konishi K (1978). An electrochemical study on the human dental enamel with special reference to isoelectric point. *J Dent Res* 57:306-312.

Pelletier C, Bouley C, Cayuela C, Bouttier S, Bourlioux P, Bellon-Fontaine MN (1997). Cell surface characteristics of *Lactobacillus casei* subsp. *casei, Lactobacillus paracasei* subsp. *paracasei,* and *Lactobacillus rhamnosus* strains. *Appl Environ Microbiol* 63:1725-1731.

Raibaud P, Caulet M, Galpin J, Mocquot G (1961). Studies on the bacterial flora of the alimentary tract of pigs. II. *Streptococci*; selective enumeration and differentiation of the dominant groups. Appl Bacteriol 24:285-291.

Rose RK, Dibdin GH, Shellis RP (1993). A quantitative study of calcium binding and aggregation in selected oral bacteria. *J Dent Res* 72:78-84.

Rose RK, Hogg SD, Shellis RP (1994). A quantitative study of calcium binding by isolated streptococcal cell walls and lipoteichoic acid: comparison with whole cells. *J Dent Res* 73:1742-1747.

Rosenberg M, Gutnik D, Rosenberg E (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9:29-33.

Sonohara R, Muramatsu N, Ohshima H, Kondo T (1995). Difference in surface properties between *Escherichia coli* and *Staphylococcus aureus* as revealed by electrophoretic mobility measurements. *Biophys Chem* 55:273-277.

Stumm W, Morgan JJ (1996). The solid-solution interface. In: Aquatic chemistry. 3rd ed. New York: Wiley-Interscience, pp. 516-613.

Sweet SP, MacFarlane TW, Samaranayake LP (1987). Determination of the cell surface hydrophobicity of oral bacteria using a modified hydrocarbon adherence method. *FEMS Microbiol Lett* 48:159-163.

van der Mei HC, Léonard AJ, Weerkamp AH, Rouxhet PG, Busscher HJ (1988). Surface properties of *Streptococcus salivarius* HB and nonfibrillar mutants: measurement of zeta potential and elemental composition with x-ray photoelectron spectroscopy. *J Bacteriol* 170:2462-2466.

van Loosdrecht MC, Lyklema J, Norde W, Schraa G, Zehnder AJ (1987). Electrophoretic mobility and hydrophobicity as measured to predict the initial steps of bacterial adhesion. *Appl Environ Microbiol* 53:1898-1901.

Verwey EJW, Overbeek JThG (1948). Theory of the stability of lyophobic colloids. Amsterdam-New York: Elsevier, pp. 1-205.

Weerkamp AH, McBride BC (1980). Adherence of *Streptococcus salivarius* HB and HB-7 to oral surfaces and saliva-coated hydroxyapatite. *Infect Immun* 30:150-158.

Weerkamp AH, Uyen HM, Busscher HJ (1988). Effect of zeta potential and surface energy on bacterial adhesion to uncoated and saliva-coated human enamel and dentin. *J Dent Res* 67:1483-1487.

Yoshida Y, Wakasa K, Kajie Y, Takahashi H, Urabe H, Satou N, *et al.* (1998). Adherent bacteria cells in five dental materials: sonication effect. *J Mater Sci Mater Med* 9:117-120.