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Biological

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Poisson Analysis of Streptococcal Bond-strengthening on Saliva-coated Enamel

ABSTRACT

The forces responsible for bond-strengthening in initial oral bacterial adhesion are unknown. Since Lifshitz-Van der Waals and electrostatic forces work instantaneously upon approach, it is hypothesized that bond-strengthening is governed by hydrogen bonding. Poisson analysis of adhesion forces observed during the retraction of bacterial probes from surfaces in atomic force microscopy can be used to analyze the nature of the adhesion forces. Streptococcal adhesion forces increased from about -0.7 to -10.3 nN when the contact time between cell surfaces and salivary films on enamel was increased from 0 to 120 sec. Initial and final adhesion forces were stronger for initial colonizers of tooth surfaces (*S. mitis*, *S. sanguinis*) than for later, more cariogenic, strains (*S. sobrinus*, *S. mutans*). Retraction curves after increased contact times showed minor peaks, representative of hydrogen bonds, and Poisson analyses indicated repulsive non-specific forces of around +0.3 nN and slightly more attractive hydrogen-bonding forces (-1.0 nN) for initial than for late colonizers (-0.8 nN).

KEY WORDS: saliva adhesion, bacteria, enamel, bond strengthening, atomic force microscopy, streptococci, biofilm.

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INTRODUCTION

Dental caries remains one of the most prevalent diseases throughout the world. Caries is a multi-factorial disease, which can be initiated by dental biofilm. The first event in dental biofilm formation is adhesion of oral bacteria to tooth surfaces (Liljemark and Bloomquist, 1996; Rosan and Lamont, 2000; Islam *et al.*, 2007). Initial bacterial adhesion of both early and later colonizers is initially reversible, but over time, adhesion becomes more irreversible. Bond-strengthening in oral bacterial adhesion has been implicated for bacterial interaction with saliva-coated glass (van der Mei *et al.*, 2008), but the nature of the forces responsible for oral bacterial bond-strengthening with salivary coatings has not yet been determined.

Bacterial adhesion forces with substratum surfaces can be measured by atomic force microscopy (AFM) (Thio and Meredith, 2008), and generally include long-range, attractive Lifshitz-Van der Waals and electrostatic forces as well as short-range hydrogen bonding (Hermansson, 1999). Lifshitz-Van der Waals and electrostatic forces are non-specific and operate instantaneously upon approach of the interacting surfaces. Hydrogen bonding, in contrast, requires stereo-chemistry between the interacting molecular groups and removal of interfacial water. The adhesion forces measured from the AFM retraction force-distance curves are characterized by a main force at close approach and a finite number of minor forces at random distances farther from the surface, obeying a Poisson distribution (Abu-Lail and Camesano, 2006). Since the minor force peaks develop only after prolonged contact between a bacterial cell surface and a substratum surface, we hypothesize that the formation of these minor peaks causes bacterial adhesion to become irreversible. The nature of the adhesion force can be decoupled into a so-called non-specific component and hydrogen-bonding forces by Poisson analysis of the force distribution (Barlow, 1989), as explained in more detail below.

The nature of the forces responsible for oral streptococcal bond-strengthening on saliva-coated enamel has never been studied. Therefore, the aim of this study was to determine the nature of the forces responsible for streptococcal bond-strengthening to saliva-coated enamel by Poisson analysis of adhesion force distributions, measured by AFM. Bond-strengthening was analyzed for 4 oral streptococcal strains, 2 of which are considered initial colonizers of tooth surfaces, and 2 strains that are later colonizers and considered to be cariogenic.

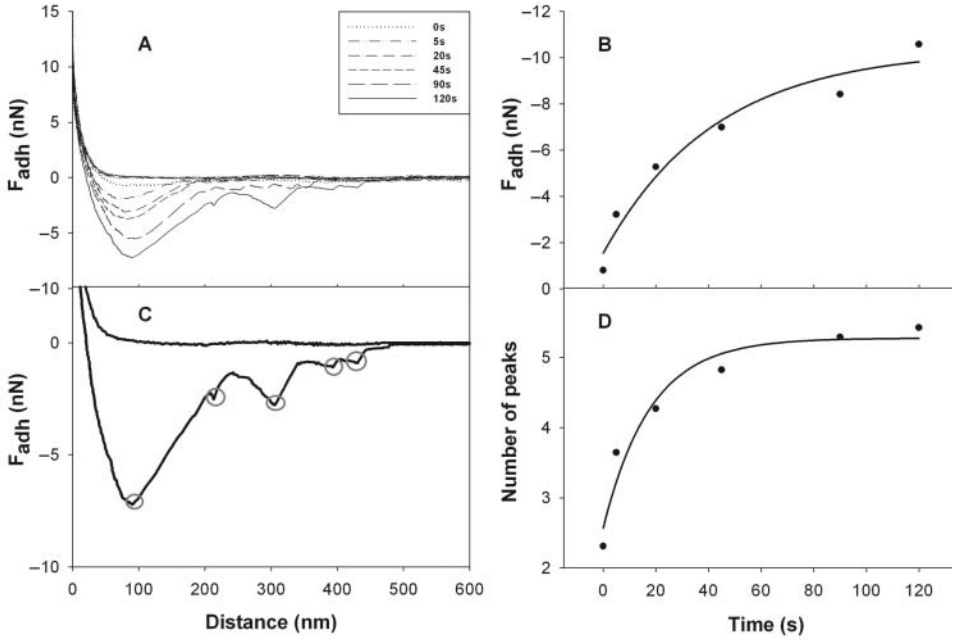

Figure. Example of bond strengthening between *S. sanguinis* ATCC10556 and saliva-coated enamel. (**A**) AFM force-distance retraction curves after different surface delay times. (**B**) Increase in maximum adhesion force as a function of the surface delay time according to an exponential rise to maximum (correlation coefficient $R^2 = 0.98$). (**C**) Indication of minor adhesion peaks in the retraction force-distance curve obtained after 120 sec of surface delay. (**D**) Increase in the number of minor adhesion peaks as a function of the surface delay.

MATERIALS & METHODS

Bacterial Cultures

Four oral bacterial strains were included in this study, namely, two early colonizers, *i.e.*, *Streptococcus mitis* BMS and *Streptococcus sanguinis* ATCC10556; and two later colonizers, also recognized as cariogenic, *i.e., Streptococcus sobrinus* HG1025 and *Streptococcus mutans* ATCC700610 (De Soet *et al.*, 1991; Li *et al.*, 2004). Streptococci were pre-cultured in Todd-Hewitt broth (Oxoid, Basingstoke, UK) for 24 hrs and inoculated into a main culture for 16 hrs at 37°C in ambient air. Bacteria were har-vested by centrifugation (5 min, 5000 *g*, 10°C), and washed twice with demineralized water. Finally, bacteria were re-suspended in demineralized water and sonicated intermittently to break bacterial aggregates in an ice/water bath for 3 x 10 sec at 30 W.

Saliva-coated Enamel Preparation

Human whole saliva was collected and prepared as has been previously described (van der Mei *et al.*, 2008) and reconstituted in adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride, and 1 mM calcium dichloride, pH 6.8) at a concentration of 1.5 mg/L. All volunteers gave their informed consent to saliva donation, in agreement with the policies of the Ethics Committee at the University Medical Center Groningen.

Enamel slabs were cut from labial surfaces of bovine incisors. An incisor was first ground under running tap water, with 220- to 1200-grit sandpaper, into 0.6 x 0.6 cm^2 blocks with a thickness of 2 mm, and subsequently micropolished on a polishing pad with wet 0.05-µm alumina particles (Buehler Ltd., Lake Bluff, IL, USA) for 3 min. Finally, enamel surfaces were cleaned by 2 min of sonication in a 35-kHz ultrasonic bath (Transsonic TP 690-A, Elma, Germany), and thoroughly rinsed with demineralized water.

We created salivary conditioning films by immersing the enamel slabs into the reconstituted saliva for 16 hrs at 20°C. All saliva-coated enamel slabs were dipped 3x in demineralized water after immersion and immediately used for AFM measurements.

AFM Measurements

Streptococci from suspension were immobilized on tipless AFM cantilevers (Ultrasharp, µ-Masch, Tallinn, Estonia). Cantilevers were first immersed in a drop of 0.01% (w/v) poly-L-lysine (Sigma, Poole, UK) for 1 min, dried for 2 min in air, and then dipped into a drop of bacterial suspension for 1 min to allow for bacterial attachment. Each thus-prepared bacterial AFM probe was used immediately for further measurement.

All AFM measurements were performed in a Dimension 3100 system (Nanoscope III, Digital Instruments, Woodbury, NY, USA) in the contact mode at room temperature in adhesion buffer, at a scan rate of 0.5 Hz, ramp size 1.5 µm, and trigger threshold of 1 V. Retraction of the bacterial probe from a saliva-coated enamel surface was done after different surface delays, ranging from 0 to 120 sec. We collected 45 force-distance curves, measured with 9 bacterial probes prepared from 3 separate bacterial cultures, for each surface delay on randomly selected positions on the saliva-coated enamel surface.

To check the integrity of the bacterial probe and the streptococcal cell surface, as well as the absence of cell-surface contamination by salivary proteins, we conducted two control experiments:

(1) We took scanning electron micrographs regularly to confirm the integrity of the bacterial probe after measurements. No force-distance curves had to be discarded due to visual damage to the bacterial probe.

(2) Adhesion forces with 0 sec surface delay were measured at the onset of each measurement cycle (from 0 to 120 sec surface delays) and again after completion of a cycle. Whenever the maximum adhesion forces at t = 0 sec and after the completion of a cycle of 120 sec did not coincide

Table 1. Maximum Adhesion Forces and Number of Peaks for 0 sec Surface Delay Time ($F_{adh,0}$ and N_0) and after Bond-strengthening ($F_{adh,\infty}$ and N_∞), Together with the Characteristic Time Constants, τ, Associated with Bond-strengthening According to Each Parameter

	F_{adh} (nN)			Number of Peaks		
Bacterial Atrain	$F_{adh,0}$ (nN) [1]	$F_{adh,\infty}$ (nN) [1]	τ (s) [2]	N_0 [2]	N_∞ [2]	τ (s) [2]
S. mitis BMS	–1.1 ± 0.5	–7.0 ± 1.1	24 ± 7	1.8 ± 0.3	3.8 ± 0.7	28 ± 17
S. sanguinis ATCC10556	–1.5 ± 0.7	–10.3 ± 2.1	43 ± 16	2.5 ± 0.3	5.3 ± 0.7	18 ± 7
S. sobrinus HG1025	–0.7 ± 0.1	–4.3 ± 0.2	51 ± 5	1.8 ± 0.3	4.0 ± 0.6	20 ± 9
S. mutans ATCC700610	–0.8 ± 0.3	–6.0 ± 0.7	27 ± 6	2.2 ± 0.3	4.5 ± 0.8	33 ± 20

[1] By convention, attractive forces are indicated as negative forces. F_{adh} were presented as median ± SE.
[2] Number of adhesion peaks in one force-distance curve. N and τ are presented as mean ± SE.

within 1 nN, data from that measurement cycle were discarded and a new bacterial probe prepared.

Calculation of Adhesion Forces and Poisson Analysis

We calculated adhesion forces after each surface delay time from the AFM deflection data, using $F = K_{sp} \times D$, in which K_{sp} is the spring constant and D is the deflection of the cantilever. We determined the spring constant of each cantilever experimentally, using the thermal method for each experiment (Burnham *et al*., 2003).

The maximal adhesion force in each force-distance curve $F_{adh}(t)$, in which t represents the surface delay time, was fitted with the use of an exponential rise to maximum function:

$$F_{adh}(t) = F_{adh,0} + (F_{adh,\infty} - F_{adh,0})\left(1 - \exp\left\{-\frac{t}{\tau}\right\}\right) \quad (1)$$

where $F_{adh,o}$ and $F_{adh,\infty}$ are the maximum adhesion after 0 sec surface delay time and after bond strengthening, and τ is the characteristic time needed for strengthening. In addition, the number of minor force peaks disrupted after each surface delay was enumerated as well (see Figs. A, C for examples) and analyzed analogously to an exponential rise to maximum (Fig. D), as described above.

Since the adhesion forces measured obeyed a Poisson distribution, the adhesion force can be expressed as:

$$P(F) = (F_{av})^n \times \frac{\exp(-F_{av})}{n!} \quad (2)$$

with $P(F)$ the possibility that an adhesion force (F) will occur, F_{av} the average of all adhesion forces, and n the total number of adhesion forces included. The total adhesion force is comprised of a main peak due to non-specific forces and hydrogen bonding and a variable number of minor peaks constituted by individual hydrogen bonds according to:

$$F = n_{H-bond}F_{H-bond} + F_{Non-specific} \quad (3)$$

where $F_{H\text{-}bond}$ and $F_{Non\text{-}specific}$ represent the contributions of hydrogen bonding and non-specific interaction forces (*i.e*., Lifshitz-Van der Waals and electrostatic forces) to the adhesion force, respectively, and $n_{H\text{-}bond}$ is the number of bonding events. Based on Eqs. 2 and 3, the relationship between the force average (μ_F) and variance (σ_F^2) of all adhesion events can be presented as:

$$\sigma_F^2 = \mu_F F_{H-bond} - F_{H-bond}F_{Non-specific} \quad (4)$$

According to Eq. 4, a plot of the variance (σ_F^2) *vs*. the force average (μ_F) yields a straight line. Linear regression of σ_F^2 *vs*. μ_F directly decouples the adhesion force into a hydrogen bonding force, $F_{H\text{-}bond}$, and the non-specific adhesion force, $F_{Non\text{-}specific}$.

A detailed example of the Poisson analysis of a force-distance curve is given in the Appendix.

Statistics

The number of peaks disrupted upon retraction N and the calculated surface delay times τ were normally distributed and are presented as mean ± SE. Comparisons of N and τ among different bacterial groups were performed with ANOVA. Adhesion forces F_{adh}, however, were not normally distributed and are presented as median ± SE and were compared by non-parametric analyses, *i.e.,* a Kruskal-Wallis test, followed by Dunn's multiple-comparison *post hoc* analysis, when overall differences were significant. Differences were considered significant when $P < 0.05$.

RESULTS

Force-Distance Curves and Bond-strengthening

An example of the bond-strengthening between *S. sanguinis* ATCC10556 and saliva-coated enamel is given in the Fig. AFM force-distance curves clearly showed stronger adhesion force maxima when the surface delay time increased (Fig. A), and this increase followed an exponential rise to maximum (Fig. B). Concurrent with the increase in the adhesion force maximum at each surface delay time was the development of multiple minor adhesion forces (Fig. C), the number of which also obeyed an exponential rise to maximum over time (Fig. D).

Table 2. Hydrogen-bonding ($F_{H\text{-}bond}$) and Non-specific Forces ($F_{Non\text{-}specific}$) of the 4 Different Bacterial Strains Involved in This Study with Saliva-coated Enamel, Obtained by Poisson Analysis of Retraction Force-Distance Curves after 120 sec of Surface Delay[1]

Bacterial Strains	$F_{H\text{-}bond}$ (nN)	$F_{Non\text{-}specific}$ (nN)
S. mitis BMS	–1.0 ± 0.2	0.3 ± 0.1
S. sanguinis ATCC10556	–1.1 ± 0.2	0.3 ± 0.1
S. sobrinus HG1025	–0.8 ± 0.1	0.3 ± 0.1
S. mutans ATCC700610	–0.8 ± 0.2	0.4 ± 0.1

[1] Forces are presented as median ± SE.

Initial adhesion forces were significantly weaker in all streptococcal strains than after bond-strengthening (Table 1), which occurred over a time scale of several tens of seconds. Both initial as well as final adhesion forces were stronger for the two initial colonizers, *S. mitis* and *S. sanguinis,* than for the later colonizers, *S. sobrinus* and *S. mutans*. Also, the number of minor adhesion peaks increased significantly over time, from around 2 to 5, which also occurred over a time scale of several tens of seconds.

Decoupling of Adhesion Forces by Poisson Analysis

Adhesion forces after bond-strengthening were decoupled (Table 2) into a hydrogen-bonding ($F_{H\text{-}bond}$) and a non-specific force ($F_{Non\text{-}specific}$). The hydrogen-bonding forces $F_{H\text{-}bond}$ were attractive for all 4 streptococcal strains involved, while the non-specific forces $F_{Non\text{-}specific}$ were positive, indicating repulsion. Initial colonizers adhered to saliva-coated enamel through significantly stronger hydrogen-bonding forces $F_{H\text{-}bond}$ than the later colonizing, cariogenic streptococci. There were no significant differences among the strains in the non-specific repulsive force $F_{Non\text{-}specific}$. Note that the force decoupling pertains only to the situation after bond-strengthening (Table 2). The total contribution of the hydrogen-bonding force, however, increased during bond-strengthening, as can be deduced from the increase in the number of minor peaks over time (Fig. C and Table 1), each representing an individual hydrogen bond with force $F_{H\text{-}bond}$.

DISCUSSION

Streptococcal adhesion to saliva-coated enamel changes from initially reversible to more irreversible through the progressive involvement over time of hydrogen-bonding forces. This strengthening of streptococcal adhesion forces could be demonstrated by AFM. Poisson analysis of the forces measured allowed for identification of the forces responsible for bond-strengthening as hydrogen-bonding forces. Interestingly, initial colonizers of tooth surfaces *in vivo* interacted through stronger individual hydrogen bonds with saliva-coated enamel than later, more cariogenic colonizers. Furthermore, they interacted through a higher number of hydrogen bonds. This resulted in an almost two-fold-stronger adhesion force after bond-strengthening for initial colonizers as compared with later colonizers, and attests to the stronger interaction with saliva-coated surfaces that initial colonizers should, by definition, possess.

The non-specific forces obtained by Poisson decoupling were repulsive (+ 0.3 nN). At first glance, this may seem surprising, since virtually all thermodynamic analyses (Van Oss, 1995) of bacterial adhesion to surfaces indicate favorable Lifshitz-Van der Waals attraction that overrules the possible existence of electrostatic repulsion in the secondary interaction minimum (Hermansson, 1999). In this respect, it must be realized, however, that the contact between the streptococcal cell surface and the saliva-coating in AFM is a forced contact beyond the interaction minimum, usually located around 5-10 nm. Contact beyond the interaction minimum is required in AFM to estimate "zero" distance between the interacting surfaces and is associated with non-specific repulsion. A Poisson analysis of multiple adhesion peaks in the interaction of *Escherichia coli* with silicon nitride AFM tips was recently performed, and a repulsive non-specific force after decoupling of +0.16 nN was found, which is slightly smaller than the non-specific force found for streptococcal adhesion to a saliva-coating (Abu-Lail and Camesano, 2006). Also, the individual hydrogen-bonding force identified was smaller than in our study, and amounted to -0.13 nN. It is currently uncertain whether this is due to the fact that those experiments were carried out with a small AFM tip *vs.* a bacterial cell surface, while we used a much larger streptococcal probe *vs.* a saliva-coating. Alternatively, it is unclear whether decoupling by Poisson analysis truly yields an estimate of a single hydrogen-bond force, or whether it determines the total hydrogen force originating from a single characteristic molecular moiety on the bacterial cell surface. The latter explanation may be more plausible in light of the fact that a hydrogen-bonding force of -0.70 nN has been obtained with a staphylococcal probe *vs.* glass (Boks *et al.*, 2008). Thus, different bacterial strains and species may adhere through their own characteristic, hydrogen-bonding molecular moieties. This is also the basis for specific interactions from a microbiological point of view (Gibbons *et al.*, 1985). They describe specific interactions as molecular recognition between ligand and receptor molecules, which operate over spatially well-confined, stereo-chemical regions, established, for instance, by interactions between acid, electron-accepting and basic, electron-donating groups or oppositely charged domains, at close approach (up to several nanometers).

The development of hydrogen bonds requires, first, the removal of interfacial water from between the interacting surfaces, while, second, hydrogen bonding involves a stereo-chemistry between the interacting groups. Both processes clearly take time, and this study indicated that bond-strengthening requires several tens of seconds. No distinction between initial and later colonizers can be made on the basis of the time required for bond-strengthening. There appears to be no relation between the characteristic time for bond-strengthening and the development of multiple individual adhesion bonds. This might be due to the fact that the total adhesion force depends on interplay between the number of bonds developing and their magnitude.

In summary, this paper has demonstrated that the important transition from reversible to more irreversible adhesion of oral streptococci to saliva-coated enamel is mediated by the progressive involvement of hydrogen bonds. Both initial as well as final adhesion forces with saliva-coated enamel were stronger for initial colonizers of tooth surfaces *in vivo* than for later, more cariogenic strains, due to both slightly more and more attractive individual hydrogen-bonding forces (-1.0 nN) for initial than for late colonizers (-0.8 nN).

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