Published in final edited form as:

Biofouling. 2011 May ; 27(5): 543–559. doi:10.1080/08927014.2011.584129.

Atomic force microscopy investigations of heterogeneities in the adhesion energies measured between pathogenic and non-pathogenic *Listeria* species and silicon nitride as they correlate to virulence and adherence

Bong-Jae Park and Nehal I. Abu-Lail*

Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA 99164-2710, USA

Abstract

Atomic force microscopy (AFM) was used to probe heterogeneities in adhesion energies measured between pathogenic and non-pathogenic species of *Listeria* and silicon nitride in water at four levels. Adhesion energies were quantified on individual bacterial cells (cell level), bacterial cells that belonged to an individual *Listeria* strain but varied in their cultures (strain level), bacterial cells that belonged to an individual *Listeria* species but varied in their strain type (species level) and on bacterial cells that belonged to the *Listeria* genus but varied in their species type (genus level). To quantify heterogeneities in the adhesion energies, a heterogeneity index was defined based on quantified standard errors of mean. At the cell level, spatial variations in the adhesion energies were not observed. For the strain, species and genus levels, the heterogeneity index increased with increase in the adhesion energies. At the species level, heterogeneity index increased with strain virulence.

Keywords

Adhesion energy; AFM; Femto-Joule; *Listeria*; Heterogeneity; Species and Bacteria

Introduction

AFM has been widely employed in the investigations of microbial surfaces' properties and interactions (Abu-Lail and Camesano 2003; Nunez, Martin et al. 2005; Abu-Lail and Camesano 2006; Abu-Lail, Liu et al. 2007). During retraction, knowledge about adhesion strength that govern the interactions of bacteria with a surface or interest (Lower, F. et al. 2001; Ahimou, Denis et al. 2002; Abu-Lail and Camesano 2003; Vadillo-Rodriguez, Busscher et al. 2003; Arce, Avci et al. 2004; Emerson and Camesano 2004; Alsteens, Dague et al. 2007; Mei, Ren et al. 2009) can be acquired in forms of adhesion forces (Ahimou, Denis et al. 2002; Abu-Lail and Camesano 2003; Abu-Lail and Camesano 2003; Vadillo-Rodriguez, Busscher et al. 2003; Emerson and Camesano 2004; Abu-Lail and Camesano 2006; Abu-Lail, Liu et al. 2007; Alsteens, Dague et al. 2007; Mei, Ren et al. 2009) or adhesion energies (Lower, F. et al. 2001; Arce, Avci et al. 2004; Cox 2007). Although both ways have been reported in the literature (Lower, F. et al. 2001; Ahimou, Denis et al. 2002; Abu-Lail and Camesano 2003; Abu-Lail and Camesano 2003; Vadillo-Rodriguez, Busscher et al. 2003; Arce, Avci et al. 2004; Emerson and Camesano 2004; Abu-Lail and Camesano 2006; Abu-Lail, Liu et al. 2007; Alsteens, Dague et al. 2007; Cox 2007; Mei, Ren et al.

*Corresponding Author: Nehal I. Abu-Lail, Ph.D., nehal@wsu.edu.

2009), the use of adhesion forces to represent bacterial adhesion is much more common. Quantifying bacterial adhesion in terms of forces is especially important when specific interactions such as ligand-receptor interactions are of interest. In comparison, the use of adhesion energy as a measure of bacterial interactions with surfaces offers a unique and comprehensive alternative way to the use of nanoscale adhesion forces (Lower, F. et al. 2001; Arce, Avci et al. 2004; Cox 2007). This is mainly due to the ease of computing the adhesion energy as the area under the retraction curve. As a result of such uniform criterion in computing adhesion energies, the adhesion energy analysis can be easily automated and bacterial interaction results can be compared among different research groups and across various microbes.

However, irrespective of whether adhesion forces or adhesion energies are to be used to quantify bacterial adhesion to surfaces, the data usually suffer from large heterogeneities that make data interpretation generally challenging (Abu-Lail and Camesano 2003). Heterogeneities in AFM measurements of bacterial adhesion can be of chemical, physical, biological or practical origins (Camesano and Abu-Lail 2002; Abu-Lail and Camesano 2003; Abu-Lail and Camesano 2003; Abu-Lail and Camesano 2006). For example, cells might vary from each other significantly depending on their growth conditions (Unnikrishnan, Cohen et al. 1999; Lower, F. et al. 2001; Meylheuc, Giovannacci et al. 2002; Carrasco, Garcia-Gimeno et al. 2006) and AFM tips may contact different molecules on the bacterial surface in a stochastic manner (Camesano and Abu-Lail 2002; Ubbink and Schar-Zammaretti 2007; Francius, Lebeer et al. 2008; Gaboriaud, Gee et al. 2008; Ma, Winslow et al. 2008). To describe heterogeneities in AFM measurements of bacterial adhesion, several statistical tools and methods have been used in the literature. Examples include reporting the adhesion data using more than one statistical measure such as the mean, mode, median and standard deviation of the population of adhesion affinities (Busscher, van de Belt-Gritter et al. 2007; Park, Haines et al. 2009). Another approach relies on fitting the distributions of the adhesion affinities to different dynamic peak functions (Abu-Lail and Camesano 2003; Abu-Lail and Camesano 2006; Park, Haines et al. 2009). In addition, researchers started to investigate the whole bacterial surface by performing bacterial measurements using a grid on the bacterial surface (Ahimou, Denis et al. 2002; Alsteens, Dague et al. 2007; Alsteens, Dupres et al. 2008; Francius, Lebeer et al. 2008). However, systematic studies that investigate heterogeneities across a larger number of cells that belong to different cultures, strains or species are still lacking mainly due to the long experimental time needed for the measurements to be carried and for the analysis to be performed.

Motivated by the lack of such studies, the adhesion energies were quantified using AFM between a model inert surface of silicon nitride and a wide array of *Listeria* cells that belonged to different *Listeria* species. We have systematically investigated magnitudes and heterogeneities in the adhesion energies at four different levels (cellular, strain, species and genus levels). The heterogeneities were quantified using a new approach that depends on normalizing the error in the measurements with respect to each other.

To perform our measurements across bacterial cells that represent a whole genus, the genus *Listeria* was chosen due to the limited number of species that belong to the genus (Farber and Peterkin 1991; Allerberger 2003). The *Listeria* genus is also unique in that it contains both pathogenic and non-pathogenic species as well as virulent and avirulent strains. To our knowledge, this is the first study that quantifies the adhesion energies and their heterogeneities for bacterial cells that represent all species of a whole bacterial genus.

The genus *Listeria* is comprised of the following six species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii* and *L. grayi* (Farber and Peterkin 1991). Among the *Listeria* species, *L. monocytogenes* is the only one that is capable of causing

human infections while *L. ivanovii* is capable of causing animal infections (Vazquez-Boland, Kuhn et al. 2001). The other *Listeria* species are non-pathogenic species (Vazquez-Boland, Kuhn et al. 2001). Due to the health risks associated with *L. monocytogenes* infections (Bottone and Namdari 1995), the virulence mechanisms of this *Listeria* species are well studied (Vazquez-Boland, Dominguez-Bernal et al. 2001). *L. monocytogenes* has thirteen recognized serotypes of which mainly *L. monocytogenes* isolates with 1/2 a, 1/2 b or 4b serotypes were found to cause infections (Lukinmaa, Aarnisalo et al. 2004). With few exceptions, strains with other serotypes can be largely considered as avirulent strains (Borucki, Gay et al. 2005).

Materials and Methods

Bacterial Cultures

Table 1 lists all the *Listeria* bacterial strains investigated in this study. *Listeria* cells were activated by growing in brain heart infusion broth (BHIB) adjusted to a pH value of 6.0 using 2N HCl for twelve hours at 30°C on a shaker rotating at 150 rpm (Carrasco, Garcia-Gimeno et al. 2006). Following the 12 hours, 1% of activated culture was transferred into 20 ml of BHIB and grown at the conditions above until the late exponential phase of growth. Cells were harvested when the absorbance for each strain at 600 nm reached a constant value that correlated well with the number of single colonies enumerated on brain heart infusion agar plates incubated at 30°C for 24 hours.

AFM Force Measurements

Prior to AFM measurements, *Listeria* cells grown until late exponential phase of growth and centrifuged twice at 5525g for 10 minutes were attached to gelatin-coated mica disks according to the procedure detailed elsewhere (Doktycz, Sullivan et al. 2003). To investigate the effect of attaching bacterial cells to gelatin-coated mica disks on the cells viability, a viability assay was carried on *L. monocytogenes* EGDe cells under water post force measurements at different times. Live/Dead BacLight™ Bacterial Viability Kit (Molecular Probes, Invitrogen) was used to perform the viability test. After 1, 2, 4 and 12 hours of disks storage at 4°C, the sample disks were stained with SYTO 9 and Propidium iodide according to the manufacturer's instructions. The excitation/emission maxima are about 480/500 nm for SYTO 9 stain and 490/635 nm for the Propidium iodide stain. Briefly, the sample disks were covered with 500 µL of DI water mixed with 0.75 µL of both dyes. After 15 minutes incubation in the dark, live (green fluorescent) and dead (red fluorescent) bacterial cells were simultaneously observed under a fluorescent microscope (Leica, Germany) equipped with long-pass filter set (Chroma Technology Corp, VT). Our images indicated that all bacterial cells were alive after one hour of incubation and that the majority of bacterial cells were alive even after 12 hours of incubation. The viability test results indicated that bacterial cells do not die over an experimental time of one hour. Therefore, for the purpose of the analysis presented in this paper, death of bacterial cells during AFM force measurements will be ignored.

All AFM force measurements were performed with a PicoForce™ Scanning Probe Microscope with Nanoscope IIIa controller and extender module (Veeco Inc., Santa Barbara, CA) under water. Silicon nitride cantilevers (DNP-S, Veeco Inc., Santa Barbara, CA) were used in all experiments as the model surface because they are characterized by a surface charge similar to each of soil and glass (Abu-Lail and Camesano 2003). Both soil and glass are substrates to which pathogenic and non-pathogenic *Listeria* strains frequently attach to in nature (Astwood and Wais 1998; Allan, Yan et al. 2004) and during food processing (Thomas, King et al. 1991; Hutter and Bechhoefer 1993). The force constant of each cantilever was determined prior to force measurements from the power spectral density

of the thermal noise fluctuations in DI water (Bunduki, Zavizion et al. 1996; Ayebah, Hung et al. 2005). Force measurements were performed under water since water is considered as the main solvent in many *in vitro* environments such as those used in food-related industries where *Listeria* cells contaminate inert surfaces (Karunasagar, Krohne et al. 1993; Lahellec, Salvat et al. 1996; Engelbrecht, Dominguez-Bernal et al. 1998). Contamination of food and food surfaces is considered as the first step towards *L. monocytogenes* and *L. ivanovii* infections (Park, Haines et al. 2009). In addition, performing the force measurements in water limits the growth of cells during the experiment. This is very important as if AFM force measurements were carried under a nutrient medium, the bacterial cells will keep growing and thus the composition of their surface molecules will be highly variable and that will lead to additional heterogeneities in the adhesion measurements (Liu, Ainsworth et al. 2003).

Prior to force measurements, bacterial cell images captured using TappingMode™ under DI water at a scan speed of 1 Hz at a resolution of 256 pixels per line and 256 lines per image were used to locate the cells, ensure the cells population homogeneity and appropriate morphology. Once a bacterial cell had been located *via* topographical scanning, the oscillation of the cantilever was stopped and the extending and retracting deflection-displacement curves measured between the bacterial surface biopolymers and the silicon nitride cantilever were recorded. Force measurements were made on a bacteria-free area of the gelatin-coated mica disk before and after making the measurement on a bacterial cell. Consistency of those measurements ensured that the tip properties had not been altered by contact with the bacterial surface biopolymers. In our experiments, at least ten cells from two to three different cultures were examined for each bacterial strain or species investigated. On each cell investigated, the point and shoot feature of the AFM software was used to select fifteen locations on the bacterial cell surface to perform force measurements (Park, Haines et al. 2009). This was true except for the cell level investigations in which adhesion energies were measured between bacterial surface biopolymers and the silicon nitride cantilever using grids that varied from one location at the center of the bacterial cell to as many as 32 locations that spanned the whole bacterial cell surface. Retraction curves were measured at a rate of 580 nm/sec to minimize the hydrodynamic drag forces (Cox 2007) and a resolution of 4096 points. In general, it took less than one hour to acquire force measurements on five bacterial cells adsorbed on the same mica-gelatin coated disk.

Analysis of Retraction Curves

Retraction curves were individually analyzed to quantify the adhesion energy between bacterial cell surface biopolymers and silicon nitride in water. The adhesion energy was computed as the area under the force-distance retraction curve with the baseline taken at zero force (Figure 1). In general, force and energy are correlated such as (Cox 2007):

$$E = - \int_{h_1}^{h_2} F dh \quad (1)$$

Where E is the adhesion energy, F is the pull-off force, and h is the separation distance. The negative sign was used to convert pull-off forces into adhesion forces. To calculate the adhesion energy, the integral in equation 1 was evaluated using the Trapezoidal rule (equation 2) (Cox 2007).

$$\int_{h_1}^{h_2} F dh \approx \frac{h_2 - h_1}{n} \left[\frac{F(h_1) + F(h_2)}{2} + \sum_{k=1}^{n-1} F\left(h_1 + k \frac{h_2 - h_1}{n}\right) \right] \quad (2)$$

In equation 2, n was equal to 4096, the number of data points collected per retraction force curve and the bounds of integration (h_1 and h_2) were taken as the first and last distance points at which the retraction curve crosses the zero force axes (gray arrows in Figure 1) and varied from one curve to another. In computing the integral in equation 1, a uniform grid was always used (Kenney and Keeping 1962).

Quantifying the Heterogeneity in Adhesion Energy Measurements

The heterogeneities in the adhesion energies were quantified using a heterogeneity index (HI). HI was defined as the ratio of the standard error of mean for a given data set to the maximum standard error of mean quantified for all sets of data that belonged to a certain level treatment. For example, for each cell, 15 curves that span the whole cell surface were used to quantify the adhesion energy of the cell to silicon nitride in water. The standard error of mean of the 15 measurements taken on each cell was computed for each cell. Cells were then ranked from the one with the lowest standard error of mean (the least heterogeneous cell) to the one with the highest standard error of mean (the most heterogeneous cell). HI was then defined as the standard error of mean obtained for each cell divided by the standard error of mean obtained for the most heterogeneous cell.

Similarly, when different strain types were investigated for the *L. monocytogenes* species, the standard error of mean of all adhesion energies quantified on at least 10 cells (number of curves varied from 150 to 390 curves) was calculated. The strains were then ranked in ascending order from the strain with the lowest standard error of mean to that with the highest standard error of mean. HI was then computed for each strain as the ratio of the standard error of mean of a specific strain to that of the strain with the largest standard error of mean. Finally, when different species were investigated for the *Listeria* genus, the standard error of mean for all adhesion energies quantified on at least 10 cells (number of curves varied from 150 to 390 curves) were calculated for each species. For the *L. monocytogenes* species, although seven strains were investigated earlier, *L. monocytogenes* EGDe was chosen to represent the *monocytogenes* species. The EGDe strain was chosen because it is the most virulent among all *L. monocytogenes* strains investigated and thus represents the pathogenicity of the *L. monocytogenes* species better than other strains. For all other *Listeria* species, one strain was investigated per species. All species were then ranked in ascending order from the species with the lowest standard error of mean to that with the highest standard error of mean (Table 1). HI was then computed as the ratio of the standard error of mean of a particular species to that of the species with the highest standard error of mean. Note that HI ratios were multiplied by a 100 through out this manuscript to indicate the percentage in heterogeneity.

Statistical Description of AFM Data

The heterogeneity in the adhesion energy measurements was quantified in terms of the standard deviation of a data set. The standard deviation (σ) of a distribution of adhesion energy data with N points is given by the square root of the sample variance as shown in equation (3) (Bell, Burke et al. 1996).

$$\sigma = \sqrt{\frac{1}{N} \sum_{i=1}^N (E_i - \bar{E})^2} \quad (3)$$

Where E_i is adhesion energy of point in the distribution of adhesion energies in fJ and $\bar{E}$ is the mean of the adhesion energy values for all the population given as:

$$\bar{E} = \frac{\sum_{i=1}^N E_i}{N} \quad (4)$$

The standard deviation is a widely used measure of the variability or dispersion from the mean value of the data in a distribution. It is easy to compute algebraically and a low standard deviation value indicates that the data points in the distribution are close to the mean and a high standard deviation indicates that the data is spread out over a large range of values. The standard error of mean (σ_M) represents the standard deviation of the sampling distribution of the mean and is given by:

$$\sigma_M = \frac{\sigma}{\sqrt{N}} \quad (5)$$

Equation (5) does not assume a normal distribution and indicates that the larger the sample size, the smaller the standard error of the mean and the more statistically representative the data are (Gray 1997).

Statistical tests were used to determine if adhesion energies quantified between bacterial surface biopolymers and silicon nitride in water varied across different locations on the cell, among different cells that belonged to a certain strain type, among different strains that belonged to a species types or among different species that belonged to the *Listeria* genus. The non-parametric group comparisons Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA) available in Sigma Plot 11.0 (Jandel Scientific) was applied to the data to compare if the differences among the median values between many (three or more) groups were statistically different ($P < 0.05$). Similarly, the Mann-Whitney Rank Sum Test available in Sigma Plot 11.0 (Jandel Scientific) was applied to the data to compare if the differences among the median values between two groups were statistically different ($p < 0.05$).

Results

Heterogeneities in Adhesion Energies Probed at Cellular level

The dependence of bacterial adhesion energies on the location of force measurements on individual bacterial cells was investigated for bacterial cells that represent all *Listeria* species in water (Figure 2). For each cell investigated, force measurements were performed on the center of the cell (one measurement) as well as on 4, 6, 9, 12 and 15 locations that spatially spanned the whole cell surface. For the *L. monocytogenes* 19118 cell, 28 and 32 measurements were also performed. It has to be noted that 32 points chosen were slightly less than the ratio of the bacterial surface area to the AFM tip area which was quantified to be approximately 50. The ratio 50 was calculated by assuming the tip as a half sphere with radius 40 nm and the bacteria as a paraboloid with radius 0.4 μm and height of 500 nm (Liu, Ainsworth et al. 2003). Figure 2 represents a series of box plots that show our results on

cells that represent seven *L. monocytogenes* bacterial strains and five strains that represent other *Listeria* species. Each box plot shows data collected on one cell. Each box in the box plot is constructed using different number of measurement points on the cell surface. The boundaries of the box represent the lower (Q1) and the upper (Q3) quartiles of the adhesion energies reported on one cell from all retraction curves analyzed. The boundaries of the error bar on the box represent the minimum and maximum adhesion energies reported on one cell from all retraction curves analyzed; i.e the range of the adhesion energy measurements. The line in the box indicates the median value of the adhesion energy or the second quartile (Q2). The circular dots surrounding any box are outlier adhesion energy values. Figure 2 shows that for any cell investigated, a range of adhesion energies is generally obtained. Figure 2 shows that as the number of points investigated on the cell surface increases, the range of adhesion energy values obtained increases as well. When the data collected on each cell using a variable number of force measurements were compared, the adhesion energy values were found to be statistically similar (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p > 0.05$). This was true for cells that represented all *Listeria* species. Our results indicate that few sample measurements were sufficient to quantify the adhesion energy between the bacterial cells investigated and silicon nitride in water. Based on our results, a constant grid of 15 points was used to perform force measurements on any bacterial cell investigated. Our choice of 15 force measurements per cell was just to collect enough data for comparison and statistical purposes.

Heterogeneities in Adhesion Energies Probed at Strain level

Heterogeneities in the adhesion energies measured between silicon nitride cantilevers and at least ten cells taken from two or three different cultures that belonged to a specific *Listeria* strain were investigated (Figure 3). Twelve *Listeria* strains were investigated. The measurements collected for each strain were represented by a series of box plots in which each box represented data taken on one cell (Figure 3). For all strains investigated, the adhesion energies measured on each cell were statistically different from those measured on different cells (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p \leq 0.001$). This was true for cells that belonged to one culture as well as for cells that belonged to different cultures. Our results thus indicate that adhesion energy measurements should be performed on more than one bacterial cell as well as on cells taken from different cultures to obtain statistically representative bacterial adhesion measurements.

The heterogeneities in the adhesion energies probed at the strain level were quantified for all cells investigated in terms of a heterogeneity index (HI) as defined in the methods earlier. Each scatter graph in Figure 4 shows the relationship between the HI values and the average adhesion energy values computed for all cells that belonged to one strain. A constant relationship that can predict all the trends shown in the scatter graphs of Figure 4 for different *Listeria* strains was not found. However, the heterogeneity index was observed to increase in general with the increase in the adhesion energies reported on the cell (Figure 4). It has to be noted as well that the HI values for all cells investigated span the whole range from 0-100 indicating that cells vary in their heterogeneity levels quite a bit (Figure 4).

Heterogeneities in Adhesion Energies Probed at the Species Level

Adhesion energies were quantified between cells that represent seven *L. monocytogenes* strains and silicon nitride in water (Table 1). The strains varied in their virulence as indicated by differences in the values of their bacterial lethal dose required to kill 50% of mice in a study (LD50) (Table 1)(Park, Haines et al. 2009). Figure 5 shows the relationship between the adhesion energies quantified for all *L. monocytogenes* strains and their corresponding LD50 values. When 97 equations were compared for their abilities to fit the data in Figure 5, the logarithmic equation gave the best fit (TableCurve v1.11, Jandel

Scientific). This finding indicates that as the *in vivo* virulence of the *L. monocytogenes* strain increases, their *in vitro* adherence to inert surfaces as indicated by the adhesion energies increases as well in a logarithmic manner. This finding is consistent with our previously reported results which indicated the presence of a similar relationship between the nanoscale adhesion forces that govern the interactions of *L. monocytogenes* with silicon nitride in water and the strains LD50 (Vadillo-Rodriguez, Busscher et al. 2003; Abu-Lail and Camesano 2006; Abu-Lail, Liu et al. 2007; Atabek and Camesano 2007).

The heterogeneities in the adhesion energies probed at the species level were quantified for all strains investigated in terms of HI. Our results indicated that the most virulent *L. monocytogenes* strain (EGDe) was characterized by the highest adhesion energy and the highest standard error of mean. The computed HI values for all *L. monocytogenes* strains investigated were then plotted against two properties of bacterial cells, namely virulence and adherence to find out if relationships do exist between the two. Figure 6A shows the relationship between HI and the adhesion energy values measured between bacterial cells and silicon nitride in water. As can be seen from the Figure, a linear relationship was found between HI and the mean of the bacterial adhesion energies reported for each strain. The slope of the linear relationship shown was positive indicating that heterogeneity increases as the adhesion energy increases.

Similarly, Figure 6B shows the relationship between HI and the bacterial virulence quantified by the LD50 values. As can be seen from the Figure, a logarithmic relationship was found between HI and bacterial virulence. The negative slope of the logarithmic relationship shown in Figure 6B indicates that heterogeneity increases as LD50 values decreases or virulence increases.

Heterogeneities in Adhesion Energies Probed at the Genus Level

Adhesion energies quantified between cells that represent all six types of *Listeria* species and silicon nitride in water were investigated (Table 1). As can be seen from Table 1, the *Listeria* pathogenic species (*L. ivanovii* and *L. monocytogenes* EGDe) were associated with higher HI values compared to the non-pathogenic species (74.6 and 52.5, respectively). Although only *L. monocytogenes* EGDe was used in the genus level analysis, it can be seen from Table 1 that all other *L. monocytogenes* virulent strains were more heterogeneous when compared to other strains of non-pathogenic *Listeria* species.

The adhesion energies quantified for all *Listeria* species were investigated to check if the pathogenic and non-pathogenic species varied in the distribution and magnitudes of their adhesion energies to inert surfaces (Figure 7). In constructing Figure 7, the six virulent *L. monocytogenes* strains and the *L. ivanovii* strain were considered as pathogenic strains while the avirulent *L. monocytogenes* strain (15313) and the strains that belonged to the other *Listeria* species were considered as non-pathogenic strains. As can be seen from Figure 7, adhesion energies spanned a range for both pathogenic and non-pathogenic strains. However, the distribution of the adhesion energies for the non-pathogenic strains was narrower compared to that collected for the pathogenic ones. To describe the distribution of the adhesion energies observed in Figure 7, the log-normal dynamic peak function was fit to the data (solid lines in Figure 7). The ability of the log-normal dynamic peak function to fit the data was judged by the values of r^2 which were 0.97 and 0.99 for the pathogenic and non-pathogenic strains, respectively. According to the log-normal function, the most probable adhesion energy values for all pathogenic and non-pathogenic *Listeria* strains were 0.142 and 0.071 fJ, respectively. These values were comparable to the arithmetic mean values of the distribution of adhesion energies which were 0.132 and 0.072 fJ for the pathogenic and non-pathogenic strains, respectively. Finally, when all the adhesion energy values quantified between silicon nitride and pathogenic and non-pathogenic *Listeria* strains

in water were compared, the results were significantly different (Mann-Whitney Rank Sum Test, $p < 0.001$).

Discussion

Spatial Heterogeneities on a Single Bacterium Cell Surface

In general, AFM measurements of bacterial cell properties and interactions are mostly carried at the center of the bacterial cell or on 5-10 randomly selected locations on the top of the bacterial cell (Abu-Lail and Camesano 2003). When analyzed, retractions curves are generally looked at individually due to the complexity of interactions displayed in those curves (van der Mei, de Vries et al. 2010) as reflected by large standard deviations (Tresse, Lebret et al. 2006). With bacterial cell surfaces known to be composed of a wide variety of surface molecules (Ahimou, Denis et al. 2002), researchers in the microbial cells community started to map microbial surface properties and interactions using grids that represent the whole cellular surface. Examples include mapping the surface charge distribution and the adhesion nanoscale forces of yeast *Saccharomyces cerevisiae* using a COOH-terminated AFM cantilever under various pH conditions using a 16×16 grid over a 250 nm^2 area (Dorobantu, Bhattacharjee et al. 2008), adhesion force maps of the biodegrading bacteria *Rhodococcus erythropolis* 20S-E1-c to hydrophobic terminated AFM cantilever (Dupres, Menozzi et al. 2005), mapping of single heparin-binding haemagglutinin adhesion (HBHA) on the surface of living *Mycobacterium tuberculosis* bacterial cells using an AFM tip decorated with heparin molecules using a 1024 force measurements on 50 nm^2 area (Busscher, van de Belt-Gritter et al. 2007), mapping the adhesion forces between *Streptococcus mutans* and laminin films in buffered solutions adjusted to pH values of 5.8 or 6.8 using a 32×32 grid over areas less than a $1 \text{ }\mu\text{m}^2$ (Gaboriaud, Parcha et al. 2008) and mapping of zero-force heights and mechanical properties of *Shewanella putrefaciens* using a 10×10 force-volume grid recorded using a silicon nitride cantilever in a 100 mM NaNO_3 with neutral pH (Dupres, Menozzi et al. 2005). Although the studies above are very interesting, a question on how large the size of the grid needs to be to statistically represent the bacterial surface remains to be answered.

To address the question above, the size of the grid on the bacterial cell was varied from one point to 32 points. The adhesion energies between the bacterial cell surface biopolymers and silicon nitride in water were then calculated. Our results indicated that the size of the grid did not influence the overall adhesion energy quantified between the bacterial cell and silicon nitride in water for the 12 strains investigated. Therefore, mapping cell surfaces might be important for studies that involve determination of the location of certain ligands or receptors on the bacterial cell surface (Ahmed, Nakagawa et al. 2000) or for studies seeking fundamental understanding of the role of localized charge domains on controlling bacterial adhesion to surfaces (Atabek and Camesano 2007; Vadillo-Rodriguez, Beveridge et al. 2008; Park and Abu-Lail 2010). However, for studies that aim at quantifying the bacterial adhesion to inert surfaces specifically, a low number of measurement points on the bacterial cell surface seems to be sufficient to statistically represent interactions between an individual cell surface and a model inert surface.

Cell-Cell Heterogeneities

Due to the time associated with performing AFM experiments on bacterial cells, a limited number of bacterial cells is generally investigated (Camesano and Abu-Lail 2002; Abu-Lail and Camesano 2006; Mei, van der Mei et al. 2009). With few exceptions (Abu-Lail and Camesano 2003; Liu, Black et al. 2006; Yongsunthon and Lower 2006), cell to cell contributions to the bacterial adhesion measurements are usually not discussed in the literature. Generally, the data collected from all cells get addressed in a comprehensive

manner and a histogram that represents variability in the measurements is generally reported for the ensemble of cells (van der Mei, de Vries et al. 2010). In this study, the question of how bacterial adhesion energy measurements vary from one cell to another and between cells that were harvested from the same culture or harvested from different cultures was asked. To address the question above, 10-26 cells harvested from two to three different cultures for each of the 12 *Listeria* strains studied here were investigated. For all strains investigated, our results indicated that cells vary significantly in their adhesion energies irrespective of whether they belonged to one or different cultures. Our results thus indicate that it is very important in AFM bacterial adhesion measurements to investigate bacterial cells from different cultures. Cell to cell variability might even become more important to account for in clinical investigations where specific growth conditions of bacterial cells are not well controlled. The number of cells to be investigated in each study will vary depending on the purpose of the measurements and the type of bacterial strains used. As a rule of thumb, the number of cells to investigate should be sufficient to obtain statistically representative quantitative bacterial adhesion measurements. Our results are consistent with a previous study in which the authors concluded that investigating bacterial cells from different cultures is more important to the reliability of the bacterial adhesion measurements than investigating variable number of locations on a bacterial cell surface with less cultures (Boonaert, Dufrene et al. 2001; da Silva and Teschke 2003; Abu-Lail and Camesano 2006).

Strain-Strain Heterogeneity

Investigating a range of bacterial strains using AFM is a difficult task because it requires significant time for data collection and analysis. In this study, we attempted to investigate the differences in adhesion energies between seven bacterial strains that belonged to a certain bacterial species (*L. monocytogenes*) and varied in their *in vivo* virulence characteristics. Our results indicated that the adhesion energies quantified between *L. monocytogenes* strains that varied in virulence and silicon nitride in water correlate well with the LD50 values reported for the strains in mice (Figure 5). Although the logarithmic correlation plotted in Figure 5 was not the only function capable of fitting the data, we have chosen this model for simplicity and because logarithmic functions are generally used to report bacterial numbers and various properties (Park, Haines et al. 2009). Previously, we have shown a similar correlation between LD50 and nanoscale adhesion forces (Vazquez-Boland, Kuhn et al. 2001). Our results suggest that virulent strains are more capable of attaching themselves to inert surfaces *in vitro* compared to avirulent strains. Although enhanced binding of virulent strains to inert surfaces compared to the avirulent strains can be one way by which virulent strains concur inert surfaces in their route towards infections (Kalmokoff, Austin et al. 2001), studies in the literature correlating virulence of *L. monocytogenes* with their macroscale adhesion to inert surfaces such as stainless steel (Tresse, Lebreton et al. 2006) and polystyrene (van der Mei, de Vries et al. 2010) have been conflicting. The conflicting results in the literature point towards the important role of high-resolution studies such as AFM in quantifying bacterial adhesion to inert surfaces and in determining possible relationships between bacterial adherence and virulence. However, one barrier to overcome in AFM measurements is the interpretation of the heterogeneities in the AFM force measurements.

To interpret heterogeneities in the AFM adhesion measurements, researchers sometimes go beyond basic statistical analysis in efforts to describe such heterogeneities. Examples include fitting the histograms of the distribution of adhesion forces or energies to dynamic peak fitting functions such as Gaussian, normal, Poisson and Weibull functions (Vazquez-Boland, Dominguez-Bernal et al. 2001). Although using all the statistical tools above is interesting and useful, it does not address how strains compare in their heterogeneities to

each other. In this study, we have defined a heterogeneity index that can be used to determine how each strain compares to another strain in heterogeneity. The heterogeneity index (HI) values defined for the different *L. monocytogenes* strains were correlated with bacterial virulence and adherence (Figures 6A and 6B). Both figures indicate that the HI values increase with the increase in the adhesion energies and the strains' virulence represented by LD50.

Our results which suggest the presence of a relationship between heterogeneity and virulence and adherence of *L. monocytogenes* strains agree well with previously published reports. First, virulent strains of *L. monocytogenes* are known for their ability to express a larger set of surface proteins compared to avirulent strains (Liu, Ainsworth et al. 2003). For example, it is well accepted that virulent *L. monocytogenes* strains express listeriolysin-O (LLO), a surface protein that is known as a virulence vector for *L. monocytogenes* and has a specific role in the microbe internalization route *in vivo* while avirulent *L. monocytogenes* strains such as ATCC 15313 does not express LLO (van der Mei, de Vries et al. 2010). As the surface of a microbe increases the variety of proteins displayed on its surface, the heterogeneities in adhesion measurements are expected to increase. Similarly, if the microbe expresses more molecules on its surface, the number of molecules that will interact with an AFM cantilever will increase leading to a higher adhesion energies quantified. Therefore, the fact that virulent *L. monocytogenes* strains express more proteins on their surfaces will potentially lead to larger adhesion energies and larger heterogeneities compared to the avirulent strains.

Several studies in the literature realized the importance of investigating various types of bacterial strains to generalize particular conclusions. For example, adhesion forces were measured between AFM tips that varied in geometry, chemistry or biology and a waterborne pathogen, *Raoultella terrigena* ATCC33527, pathogenic *Staphylococcus aureus* 835 and *Lactobacillus crispatus* probe and a urogenital pathogen *S. aureus* MN8. The distribution in adhesion force measurements was described using a Weibull analysis (van der Mei, de Vries et al. 2010). The authors concluded that the Weibull distribution is a useful new way in describing the heterogeneities in AFM adhesion force data (Strauss, Kadilak et al. 2010). In another study, the adhesion of nine O-antigen expressing *E. coli* strains and three control strains that lacked O-antigen to an antimicrobial peptide cecropin P1 were quantified in controlled buffer solution (Strauss, Kadilak et al. 2010). According to the authors, a relationship was found between the strength of adhesion forces measured by AFM and the length of the bacterial LPS molecules (Cabanes, Dehoux et al. 2002).

Species-Species Heterogeneities

To our knowledge, this is the first study in the literature that probes bacterial cells that represent all species of a bacterial genus at the nanoscale level. One question that we aimed at answering was do pathogenic and non-pathogenic species of *Listeria* share similar adhesion characteristics to inert surfaces. The *Listeria* species were thus categorized according to pathogenicity. *L. monocytogenes* EGDe and the *L. ivanovii* LMB 1380 were placed in the pathogenic category. Other *Listeria* species including those of *seeligeri*, *grayi*, *welshemeri* and *innocua* were placed in the non-pathogenic category. Our results indicated that the pathogenic species of *Listeria* were characterized by larger HI values compared to the non-pathogenic *Listeria* species (Table 1). In addition, the average adhesion energy for the two pathogenic species investigated was approximately two folds higher compared to that of the non-pathogenic species (0.139 and 0.076 fJ, respectively) (Table 1). This is consistent with our findings on *L. monocytogenes* strains alone which indicated that as the virulence of the *L. monocytogenes* strain increased, the adhesion values and heterogeneities increased as well.

Our findings here are consistent with variations in the surface composition of virulent and avirulent *Listeria* species. For example, *L. monocytogenes* EGDe encodes a total of 133 surface proteins which accounts for approximately 4.7% of the coding capacity of the whole bacterium genome. In comparison, 22.6% of cell-surface protein encoding genes are absent from *L. innocua* CLIP 11262, whereas only 9.5% of the total *L. monocytogenes* genes are absent from *L. innocua*, indicating that the main differences between these two species lie in their surface protein composition. The abundance of the virulent *L. monocytogenes* surface proteins in comparison to the avirulent *L. innocua* is probably a key contributor to the larger heterogeneity observed for the virulent species compared to the avirulent species. The larger surface protein composition as well is likely related to the ability of the virulent species to interact with a large variety of inert and biotic surfaces (Rocourt 1988; Howard, Harsono et al. 1992). According to DNA homology values as well as to 16S rRNA cataloguing results, *Listeria* species can be classified into two categories with all *Listeria* species being closely related and differs from *L. grayi* (Vazquez-Boland, Dominguez-Bernal et al. 2001). This can be the reason for why *L. grayi* showed higher femto-Joule adhesion energies (0.12 fJ) compared to all the other avirulent *Listeria* species (Table 1). When compared, both *L. monocytogenes* and *L. ivanovii* can cause animal infections and are typical facultative parasites. However, *L. monocytogenes* can infect a wide range of animal species, including mammals and birds while *L. ivanovii* has a narrower host range, being pathogenic mostly for ruminants (Buchrieser, Rusniok et al. 2003). This is interesting as our results indicate that *L. ivanovii* are lower in their adhesion energies compared to *L. monocytogenes*. As can be seen from Table 1, the adhesion energy of *L. ivanovii* to silicon nitride in water is lower than the adhesion energies reported for all virulent *L. monocytogenes* strains to silicon nitride in water (Table 1). This result supports our findings in general that as virulence of bacteria increases, their in vitro adherence increases as well.

Magnitudes of Adhesion Energies Quantified for *Listeria* species

As can be seen from Table 1, adhesion energies quantified in the current study for 12 *Listeria* species ranged from 0.039 fJ for *L. innocua* 415 to 0.168 fJ for *L. monocytogenes* EGDe. To describe the heterogeneities in the adhesion energies measured for all *Listeria* species, the strains that represent the various species were categorized into pathogenic or non-pathogenic strains. For example, although *L. monocytogenes* is known to be a pathogenic *Listeria* species, its 15313 strain is avirulent. Therefore, *L. monocytogenes* 15313 was lumped together with the non-pathogenic strains for the overall analysis of the adhesion energies of *Listeria* species.

Figure 7 shows the distribution of the adhesion energies for all *Listeria* pathogenic and non-pathogenic species. According to the log-normal dynamic peak function model, pathogenic species were characterized by twice as higher adhesion energies, wider distributions and more heterogeneous data compared to the non-pathogenic species (Figure 7). This is to be expected as the pathogenic species are characterized by a larger composition of surface proteins compared to the non-pathogenic species (Limpert, Stahel et al. 2001).

The log-normal dynamic peak function was chosen to fit the data because it is well suited for measurements that show more or less a skewed distribution such as AFM data. Skewed distributions are common when the variations in the measurements are large. The log-normal dynamic peak function ability to fit distributions of data in common biological mechanisms such as bacterial growth, latent periods to acquire infections and survival periods after people are diagnosed with cancer was evident in the literature (Park, Haines et al. 2009). Previously, we have shown the ability of the log-normal dynamic peak function to fit the distribution of bacterial adhesion forces quantified by AFM (Abu-Lail and Camesano 2003; Vadillo-Rodriguez and Logan 2006; Park, Haines et al. 2009; van der Mei, de Vries et al. 2010). Other models that have been used in the literature to describe various distributions

of AFM data include the normal, Gaussian, Gamma and Weibull models (Abu-Lail and Camesano 2003; van der Mei, de Vries et al. 2010).

In the literature, few studies have quantified the adhesion of bacteria to surfaces in terms of adhesion energies. When compared to such studies, the magnitudes of adhesion energies quantified between *Listeria* species and silicon nitride in water agreed well with the reported adhesion energy values for other bacterial species. For example, the adhesion energies quantified between *Shewanella oneidensis* and goethite in aerobic solutions were found to be 0.026 fJ on average after allowing the bacteria and goethite to equilibrate for 5 minutes before the AFM measurements (Postollec, Norde et al. 2006). Another example is given by the interaction energies quantified between non-co-aggregating pairs of *Actinomyces naeslundii* 147 which were found to be 0.146 fJ and 0.233 fJ in 22°C and 40°C, respectively (Tresse, Lebret et al. 2006).

Sources of Heterogeneities in AFM Bacterial Adhesion Measurements

The large variations generally reported in the adhesion forces or energies quantified between bacteria and surfaces using AFM make data interpretation challenging. The sources of heterogeneities in the AFM measurements can be physical, chemical, biological and practical sources. Physical heterogeneities can arise from the fact that the AFM cantilever can interact with variable surface molecules every time. Bacterial surfaces are known to be composed of many types of surface molecules including lipopolysaccharides, glycoproteins, lipids, surface proteins, and sometimes an additional layer of extracellular polysaccharides (Dufrene and Rouxhet 1996; Cabanes, Dehoux et al. 2002; Calvo, Pucciarelli et al. 2005). The complexity of the bacterial surface is a main reason behind the heterogeneities observed in the AFM adhesion measurements (Abu-Lail and Camesano 2006). Physical heterogeneities can also arise from variable contact areas between the AFM tip and the bacterial surface at different force measurements. Note that the AFM tip is large in size compared to the size of the bacterial surface biopolymers and thus will most likely contact a number of bacterial surface biopolymers rather than contacting one biopolymer chain (Camesano and Abu-Lail 2002). Depending on the types, charges and hydrophobicities of the molecules the AFM tip contacts, the strength of the adhesion interactions will be variable (Vadillo-Rodriguez and Logan 2006). The actual contact area between the bacterium and the AFM tip will also depend on the applied load by the cantilever as well as on the size of the asperities of the interacting surfaces. Physical contact between the bacterial molecules and the AFM cantilever thus deviates from the definition of continuum surfaces. The use of colloidal AFM probe was shown to reduce the heterogeneities observed in bacterial adhesion measurements compared to when a nanoscale size tip was used. The authors suggested that the continuum averaging of the adhesion forces over larger molecules when the colloidal tip was used resulted in lower adhesion variations compared to the localized adhesion forces obtained using a sharp AFM tip (Tresse, Lebret et al. 2006). Finally, the interactions generally reflect the nature of specific molecules on the bacterial surface. An AFM tip can contact many molecules at once as well as can contact a single molecule in more than one specific way. The heterogeneity is thus largely affected by the stochastic nature of the behavior of bacterial surface biopolymers.

Chemical heterogeneities are the result of the different types of biopolymers that are present on the bacterial surface. A cell is generally composed of many types of biopolymers that may vary in their chemical composition (Bierne and Cossart 2007). The molecules might as well vary in their conformational properties with time and thus display or hide certain chemical groups from their surfaces at the time of contact with the AFM tip. For example, annotation of the first *L. monocytogenes* genome (strain EGDe) predicted a total of 2,853 proteins, among which were 133 surface proteins that were precisely classified according to their different anchoring systems and potential structural domains (Folio, Chavant et al.

2004). Biological heterogeneities may arise from differences between cells. Cells vary from each other in the types of molecules they express on their surfaces. Although in our experiments, the phase at which cells were cultivated and the ways by which cells were washed and treated prior to AFM measurements were tightly controlled, cells were still different. It is well known that cells produce different molecules at different stages of growth (Liu, Ainsworth et al. 2003). Therefore, if cells happen to be marginally different in their phase of growth at the time of cultivation, they will behave differently. Therefore, it is critical for AFM force measurements on bacterial cells to get carried for cells with controlled age and phase of growth to minimize heterogeneities that may result from variability in the composition of bacterial surface biopolymers as a function of cellular growth. Finally, practical problems such as signal to noise ratio, funny-shaped adhesion peaks and statistical processing of the data could all lead to heterogeneities in the AFM measurements.

Conclusions

AFM was successfully used to quantify adhesion energies in water between silicon nitride and 12 different *Listeria* strains that represented all 6 *Listeria* species. The heterogeneities in the adhesion energies were probed at four different levels namely the cell, strain, species and genus levels using a heterogeneity index defined on the basis of standard error of mean. The quantified heterogeneity index values for bacterial strains were further correlated with bacterial virulence and adherence. Our results indicated the absence of spatial variations in the adhesion energies quantified at the single cell level. However, variations in the adhesion energies were evident among cells irrespective of whether cells were cultivated from a single bacterial culture or from different bacterial cultures. This was true for all strains investigated. At the species level, heterogeneities in the adhesion energies were found to increase with the increase in strain virulence represented by LD50 and with the increase in the overall adhesion energy quantified between cells that belonged to a certain bacterial strain and silicon nitride. Finally, heterogeneities in the adhesion energies measured between bacterial cells that represent all species of a bacterial genus (*Listeria*) and silicon nitride model surface in water were probed for the first time in the literature. Our results indicated that the pathogenic species of *Listeria* were characterized by larger adhesion energies to silicon nitride and by more heterogeneities as well compared to the non-pathogenic species. The approach developed here to quantify heterogeneities in bacterial adhesion measurements can be used to describe heterogeneities in AFM interaction measurements performed on viral cells, mammalian cells and even single molecules.

Acknowledgments

We would like to thank Prof. Mark Lawrence, an associate professor at the Department of Basic Sciences, the College of Veterinary medicine at Mississippi State University for providing us with the *L. monocytogenes* ATCC 15313, ATCC 19118, ATCC 19112, ATCC 19115, 874, 1002, EGDe and the *L. innocua* 415 strains used in the current study. We would like to thank also Prof. Douglas Call, an associate professor at the Department of Veterinary Microbiology and Pathology, the College of Veterinary medicine at Washington State University for providing us with the *L. ivanovii* LMB1380 and the *L. welshimeri* LMB0082 strains used in this study. We would like to thank Joshue Orellana, Fabiola Quiora, Ala' I. Abu-Lail, Sharon Cox, David G. Wyrick, Corina Chilibeck, Kaori Graybeal, Jessica Jensen and Travis Haines for help in data analysis. Finally, we would like to thank the National Science Foundation grant EEC-0823901 and the National Institutes of Health grant numbers 1R03AI077590-01A1 and 5R03AI077590-02 for financial support of this work.

References

- Abu-Lail LI, Liu Y, Atabek A, Camesano TA. Quantifying the adhesion and interaction forces between *Pseudomonas aeruginosa* and natural organic matter. Environmental Science & Technology. 2007; 41:8031–8037. [PubMed: 18186333]

- Abu-Lail NI, Camesano TA. Polysaccharide properties probed with atomic force microscopy. *Journal of Microscopy-Oxford*. 2003; 212:217–238.
- Abu-Lail NI, Camesano TA. Role of ionic strength on the relationship of biopolymer conformation, DLVO contributions, and steric interactions to bioadhesion of *Pseudomonas putida* KT2442. *Biomacromolecules*. 2003; 4:1000–1012. [PubMed: 12857085]
- Abu-Lail NI, Camesano TA. Role of lipopolysaccharides in the adhesion, retention, and transport of *Escherichia coli* JM109. *Environmental Science & Technology*. 2003; 37:2173–2183. [PubMed: 12785523]
- Abu-Lail NI, Camesano TA. The effect of solvent polarity on the molecular surface properties and adhesion of *Escherichia coli*. *Colloids and Surfaces B-Biointerfaces*. 2006; 51:62–70.
- Abu-Lail NI, Camesano TA. Specific and nonspecific interaction forces between *Escherichia coli* and silicon nitride, determined by Poisson statistical analysis. *Langmuir*. 2006; 22:7296–7301. [PubMed: 16893229]
- Ahimou F, Denis FA, Touhami A, Dufrene YF. Probing microbial cell surface charges by AFM. *Langmuir*. 2002; 18:9937–9941.
- Ahmed K, Nakagawa T, Nakano Y, Martinez G, Ichinose A, Zheng CH, Akaki M, Aikawa M, Nagatake T. Attachment of *Moraxella catarrhalis* occurs to the positively charged domains of pharyngeal epithelial cells. *Microbial Pathogenesis*. 2000; 28:203–209. [PubMed: 10764611]
- Allan JT, Yan Z, Kornacki JL. Surface material, temperature, and soil effects on the survival of selected foodborne pathogens in the presence of condensate. *Journal of Food Protection*. 2004; 67:2666–2670. [PubMed: 15633669]
- Allerberger F. *Listeria*: growth, phenotypic differentiation and molecular microbiology. *Fems Immunology and Medical Microbiology*. 2003; 35:183–189. [PubMed: 12648835]
- Alsteens D, Dague E, Rouxhet PG, Baulard AR, Dufrene YF. Direct measurement of hydrophobic forces on cell surfaces using AFM. *Langmuir*. 2007; 23:11977–11979. [PubMed: 17941657]
- Alsteens D, Dupres V, Evoy KM, Wildling L, Gruber HJ, Dufrene YF. Structure, cell wall elasticity and polysaccharide properties of living yeast cells, as probed by AFM. *Nanotechnology*. 2008; 19:384005. [PubMed: 21832565]
- Arce FT, Avci R, Beech IB, Cooksey KE, Wigglesworth-Cooksey B. A live bioprobe for studying diatom-surface interactions. *Biophysical Journal*. 2004; 87:4284–4297. [PubMed: 15377513]
- Astwood AC, Wais AC. Psychrotrophic bacteria isolated from a constantly warm tropical environment. *Current Microbiology*. 1998; 36:148–151. [PubMed: 9516542]
- Atabek A, Camesano TA. Atomic force microscopy study of the effect of lipopolysaccharides and extracellular polymers on adhesion of *Pseudomonas aeruginosa*. *Journal of Bacteriology*. 2007; 189:8503–8509. [PubMed: 17905997]
- Ayebah B, Hung YC, Frank JF. Enhancing the bactericidal effect of electrolyzed water on *Listeria monocytogenes* biofilms formed on stainless steel. *Journal of Food Protection*. 2005; 68:1375–1380. [PubMed: 16013373]
- Bell BM, Burke JV, Schumitzky A. A relative weighting method for estimating parameters and variances in multiple data sets. *Computational Statistics & Data Analysis*. 1996; 22:119–135.
- Bierne, h; Cossart, p. *Listeria monocytogenes* surface proteins: From genome predictions to function. *Microbiology and Molecular Biology Reviews*. 2007; 71:377–397. [PubMed: 17554049]
- Boonaert CJP, Dufrene YF, Derclaye SR, Rouxhet PG. Adhesion of *Lactococcus lactis* to model substrata: direct study of the interface. *Colloids and Surfaces B-Biointerfaces*. 2001; 22:171–182.
- Borucki MK, Gay CC, Reynolds J, McElwain KL, Kim SH, Call DR, Knowles DP. Genetic diversity of *Listeria monocytogenes* strains from a high-prevalence dairy farm. *Applied and Environmental Microbiology*. 2005; 71:5893–5899. [PubMed: 16204502]
- Bottone EJ, Namdari H. *Listeria-monocytogenes* infections - Laboratory investigation of an unexpected increase in incidence. *Mount Sinai Journal of Medicine*. 1995; 62:216–220. [PubMed: 7616977]
- Buchrieser C, Rusniok C, Kunst F, Cossart P, Glaser P. Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria innocua*: Clues for evolution and pathogenicity. *Fems Immunology and Medical Microbiology*. 2003; 35:207–213. [PubMed: 12648839]

- Bunduki MMC, Zavizion BA, Politis I, Donnelly CW. Virulence of culture filtrate from heat-injured and repaired *Listeria* strains: Assay on bovine mammary epithelial (MAC-T) cells. *Journal of Food Protection*. 1996; 59:932–937.
- Busscher HJ, van de Belt-Gritter B, Dijkstra RJB, Norde W, Petersen FC, Scheie AA, van der Mei HC. Intermolecular forces and enthalpies in the adhesion of *Streptococcus mutans* and an antigen I/II-deficient mutant to laminin films. *Journal of Bacteriology*. 2007; 189:2988–2995. [PubMed: 17277062]
- Cabanes D, Dehoux P, Dussurget O, Frangeul L, Cossart P. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. *Trends in Microbiology*. 2002; 10:238–245. [PubMed: 11973158]
- Calvo E, Pucciarelli MG, Bierne H, Cossart P, Albar JP, Garcia del Portilla F. Analysis of the *Listeria* cell wall proteome by two-dimensional nano-liquid chromatography coupled to mass spectrometry. *Proteomics*. 2005; 5:433–443. [PubMed: 15627966]
- Camesano TA, Abu-Lail NI. Heterogeneity in bacterial surface polysaccharides, probed on a single-molecule basis. *Biomacromolecules*. 2002; 3:661–667. [PubMed: 12099808]
- Carrasco E, Garcia-Gimeno R, Seselovsky R, Valero A, Perez F, Zurera G, Todd E. Predictive model of *Listeria monocytogenes*' growth rate under different temperatures and acids. *Food Science and Technology International*. 2006; 12:47–56.
- Cox MG. The area under a curve specified by measured values. *Metrologia*. 2007; 44:365–378.
- da Silva A, Teschke O. Effects of the antimicrobial peptide PGLa on live *Escherichia coli*. *Biochimica Et Biophysica Acta-Molecular Cell Research*. 2003; 1643:95–103.
- Doktycz MJ, Sullivan CJ, Hoyt PR, Pelletier DA, Wu S, Allison DP. AFM imaging of bacteria in liquid media immobilized on gelatin coated mica surfaces. *Ultramicroscopy*. 2003; 97:209–216. [PubMed: 12801673]
- Dorobantu LS, Bhattacharjee S, Foght JM, Gray MR. Atomic force microscopy measurement of heterogeneity in bacterial surface hydrophobicity. *Langmuir*. 2008; 24:4944–4951. [PubMed: 18355095]
- Dufrene YF, Rouxhet PG. Surface composition, surface properties, and adhesiveness of *Azospirillum brasilense* - Variation during growth. *Canadian Journal of Microbiology*. 1996; 42:548–556.
- Dupres V, Menozzi FD, Loch C, Clare BH, Abbott NL, Cuenot S, Bompard C, Raze D, Dufrene YF. Nanoscale mapping and functional analysis of individual adhesins on living bacteria. *Nature Methods*. 2005; 2:515–520. [PubMed: 15973422]
- Emerson RJ, Camesano TA. Nanoscale investigation of pathogenic microbial adhesion to a biomaterial. *Applied and Environmental Microbiology*. 2004; 70:6012–6022. [PubMed: 15466545]
- Engelbrecht F, Dominguez-Bernal G, Hess J, Dickneite C, Greiffenberg L, Lampidis R, Raffelsbauer D, Daniels JJD, Kreft J, Kaufmann SHE, Vazquez-Boland JA, Goebel W. A novel PrfA-regulated chromosomal locus, which is specific for *Listeria ivanovii*, encodes two small, secreted internalins and contributes to virulence in mice. *Molecular Microbiology*. 1998; 30:405–417. [PubMed: 9791184]
- Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*. 1991; 55:476–511. [PubMed: 1943998]
- Folio P, Chavant P, Chafsey I, Belkorchia A, Chambon C, Hebraud M. Two-dimensional electrophoresis database of *Listeria monocytogenes* EGDe proteome and proteomic analysis of mid-log and stationary growth phase cells. *Proteomics*. 2004; 4:3187–3201. [PubMed: 15378702]
- Francius G, Lebeer S, Alsteens D, Wildling L, Gruber HJ, Hols P, De Keersmaecker S, Vanderleyden J, Dufrene YF. Detection, localization, and conformational analysis of single polysaccharide molecules on live bacteria. *Acs Nano*. 2008; 2:1921–1929. [PubMed: 19206433]
- Gaboriaud F, Gee ML, Strugnell R, Duval JFL. Coupled electrostatic, hydrodynamic, and mechanical properties of bacterial interfaces in aqueous media. *Langmuir*. 2008; 24:10988–10995. [PubMed: 18512877]
- Gaboriaud F, Parcha BS, Gee ML, Holden JA, Strugnell RA. Spatially resolved force spectroscopy of bacterial surfaces using force-volume imaging. *Colloids and Surfaces B-Biointerfaces*. 2008; 62:206–213.

- Gray, A. The paraboloid. *Modern Differential Geometry of Curves and Surfaces with Mathematica*. CRC Press; Boca Raton, FL: 1997. p. 307-308.
- Howard PJ, Harsono KD, Luchansky JB. Differentiation of *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, and *Listeria seeligeri* by pulsed-field gel-electrophoresis. *Applied and Environmental Microbiology*. 1992; 58:709–712. [PubMed: 1610193]
- Hutter JL, Bechhoefer J. Calibration of atomic force microscope tips. *Review of Scientific Instruments*. 1993; 64:1868–1873.
- Kalmokoff ML, Austin JW, Wan XD, Sanders G, Banerjee S, Farber JM. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology*. 2001; 91:725–734. [PubMed: 11576310]
- Karunasagar I, Krohne G, Goebel W. *Listeria ivanovii* is capable of cell-to-cell spread involving actin polymerization. *Infection and Immunity*. 1993; 61:162–169. [PubMed: 8418038]
- Kenney, JF.; Keeping, ES. The standard deviation and calculation of the standard deviation. *Mathematics of Statistics*. Van Nostrand; Princeton, NJ: 1962. p. 77-80.
- Lahellec C, Salvat G, Brisabois A. Incidence of food contamination by *Listeria monocytogenes*. *Pathologie Biologie*. 1996; 44:808–815. [PubMed: 8977903]
- Limpert E, Stahel WA, Abbt M. Log-normal distributions across the sciences: Keys and clues. *Bioscience Biotechnology and Biochemistry*. 2001; 51:341–352.
- Liu D, Ainsworth AJ, Austin FW, Lawrence ML. Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of Medical Microbiology*. 2003; 52:1065–1070. [PubMed: 14614064]
- Liu YT, Black MA, Caron L, Camesano TA. Role of cranberry juice on molecular-scale surface characteristics and adhesion behavior of *Escherichia coli*. *Biotechnology and Bioengineering*. 2006; 93:297–305. [PubMed: 16142789]
- Lower SK, F HJM, Beveridge TJ. Bacterial recognition of mineral surfaces: Nanoscale interactions between *Shewanella* and α -FeOOH. *Science*. 2001; 292:1360–1363. [PubMed: 11359008]
- Lukinmaa S, Aarnisalo K, Suihko ML, Siitonen A. Diversity of *Listeria monocytogenes* isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. *Clinical Microbiology and Infection*. 2004; 10:562–568. [PubMed: 15191386]
- Ma H, Winslow CJ, Logan BE. Spectral force analysis using atomic force microscopy reveals the importance of surface heterogeneity in bacterial and colloid adhesion to engineered surfaces. *Colloids and Surfaces B-Biointerfaces*. 2008; 62:232–237.
- Mei L, Ren Y, Busscher HJ, Chen Y, van der Mei HC. Poisson analysis of streptococcal bond-strengthening on saliva-coated enamel. *Journal of Dental Research*. 2009; 88:841–845. [PubMed: 19767582]
- Mei L, van der Mei HC, Ren YJ, Norde W, Busscher HJ. Poisson analysis of *Streptococcal* bond strengthening on stainless steel with and without a salivary conditioning film. *Langmuir*. 2009; 25:6227–6231. [PubMed: 19284713]
- Meylheuc T, Giovannacci I, Briandet R, Bellon-Fontaine M-N. Comparison of the cell surface properties and growth characteristics of *Listeria monocytogenes* and *Listeria innocua*. *Journal of Food Protection*. 2002; 65:786–793. [PubMed: 12030289]
- Nunez ME, Martin MO, Chan PH, Duong LK, Sindhurakar AR, Spain EM. Atomic force microscopy of bacterial communities. *Environmental Microbiology*. 2005; 397:256–268.
- Park BJ, Abu-Lail NI. Variations in the nanomechanical properties of virulent and avirulent *Listeria monocytogenes*. *Soft Matter*. 2010; 6:3898–3909. [PubMed: 20871743]
- Park BJ, Haines T, Abu-Lail NI. A correlation between the virulence and the adhesion of *Listeria monocytogenes* to silicon nitride: An atomic force microscopy study. *Colloids and Surfaces B-Biointerfaces*. 2009; 73:237–243.
- Postollec F, Norde W, de Vries J, Busscher HJ, van der Mei HC. Interactive forces between co-aggregating and non-co-aggregating oral bacterial pairs. *Journal of Dental Research*. 2006; 85:231–234. [PubMed: 16498069]
- Rocourt J. Taxonomy of the genus *Listeria*. *Infection*. 1988; 16:S89–S91. [PubMed: 2458322]

- Strauss J, Kadilak A, Cronin C, Mello CM, Camesano TA. Binding, inactivation, and adhesion forces between antimicrobial peptide cecropin P1 and pathogenic *E. coli*. *Colloids and Surfaces B-Biointerfaces*. 2010; 75:156–164.
- Thomas EJG, King RK, Burchak J, Gannon VPJ. Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Applied and Environmental Microbiology*. 1991; 57:2576–2580. [PubMed: 1768130]
- Tresse O, Lebre V, Benezech T, Faille C. Comparative evaluation of adhesion, surface properties, and surface protein composition of *Listeria monocytogenes* strains after cultivation at constant pH of 5 and 7. *Journal of Applied Microbiology*. 2006; 101:53–62. [PubMed: 16834591]
- Ubbink J, Schar-Zammaretti P. Colloidal properties and specific interactions of bacterial surfaces. *Current Opinion in Colloid & Interface Science*. 2007; 12:263–270.
- Unnikrishnan M, Cohen J, Sriskandan S. Growth phase dependent expression of virulence factors in an MIT1 clinical isolate of *Streptococcus pyogenes*. *Infect. Immun*. 1999; 67:5495–5499. [PubMed: 10496938]
- Vadillo-Rodriguez V, Beveridge TJ, Dutcher JR. Surface viscoelasticity of individual Gram-negative bacterial cells measured using atomic force microscopy. *Journal of Bacteriology*. 2008; 190:4225–4232. [PubMed: 18408030]
- Vadillo-Rodriguez V, Busscher HJ, Norde W, de Vries J, van der Mei HC. On relations between microscopic and macroscopic physicochemical properties of bacterial cell surfaces: An AFM study on *Streptococcus mitis* strains. *Langmuir*. 2003; 19:2372–2377.
- Vadillo-Rodriguez V, Logan BE. Localized attraction correlates with bacterial adhesion to glass and metal oxide substrata. *Environmental Science & Technology*. 2006; 40:2983–2988. [PubMed: 16719101]
- van der Mei HC, de Vries J, Busscher HJ. Weibull analyses of bacterial interaction forces measured using AFM. *Colloids and Surfaces, B: Biointerfaces*. 2010; 78:372–375.
- Vazquez-Boland JA, Dominguez-Bernal G, Gonzalez-Zorn B, Kreft J, Goebel W. Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection*. 2001; 3:571–584. [PubMed: 11418331]
- Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, Gonzalez-Zorn B, Wehland J, Kreft J. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev*. 2001; 14:584–640. [PubMed: 11432815]
- Yongsunthorn R, Lower SK. Force spectroscopy of bonds that form between a *Staphylococcus* bacterium and silica or polystyrene substrates. *Journal of Electron Spectroscopy and Related Phenomena*. 2006; 150:228–234.

Figure 1.

Example on a retraction curve measured between AFM silicon nitride cantilever and *L. monocytogenes* EGDe in water. The gray shadowed area represents the adhesion energy. The gray arrows at the top of curve indicate the bounds of integration where the adhesion energy was computed using equations 1 and 2.

Figure 2.

Each Figure shows box plots collected for adhesion energies measured on individual bacterial cells. On each cell investigated, 1, 2, 4, 6, 9, 12 and 15 force measurements were made successively on various locations on the cell surface as shown in the images at the bottom of Figure 2. Figures are shown for 7 strains of *L. monocytogenes* and one representative strain of each of the other five types of *Listeria* species. The *L. monocytogenes* strains are arranged according to their virulence in ascending order from the lowest in virulence strain (15313) to the highest in virulence strain (EGDe). The tapping mode images at the bottom of the Figure show point and shoot AFM maps of the locations of force measurements performed on one *L. monocytogenes* 19118 cell; the only cell that was investigated as well using 24 and 28 force measurement points. On each image, the white crosses indicate the locations of force measurements. The arrows plotted on the images indicate the sequence of the force measurements. The red circle indicates the location at which the last force measurement was performed. The number at the corner of the image indicates the number of force measurements performed on the cell surface. The dimensions of the images shown are 800 nm in width, 3 μ m in length and 200 nm in height.

Figure 3.

Each Figure shows a series of box plots that represent adhesion energies quantified between silicon nitride and different cells that belonged to a certain *Listeria* strain in water. Number of cells investigated per strain varied from 10 cells to 26 cells. In each box plot, columns with the same filling represent cells that were cultivated from one culture. Up to three filling types of boxes were used to indicate cells taken from three different cultures.

Figure 4.

A series of scatter graphs of which each represents the relationship between the heterogeneity index quantified for a number of cells that belonged to one *Listeria* strain and the average adhesion energy quantified for all adhesion energies measured on each cell investigated. The error bars represent the standard error of mean in the adhesion energies quantified for each cell.

Figure 5.

The relationship between *L. monocytogenes* virulence represented by the 50% lethal dose (LD50) and the average femto-Joule adhesion energy measured between *L. monocytogenes* surface biopolymers and silicon nitride in water. The error bars represent the standard error of mean in the adhesion energy measurements on all cells that belonged to a *L. monocytogenes* strain. The solid line is the regression fit of the data described by: adhesion energy (fJ) = $-0.0098 \ln(\text{LD50}) + 0.3297$, $r^2 = 0.89$.

Figure 6.

A) The relationship between the average femto-Joule adhesion energy quantified between silicon nitride and *L. monocytogenes* cells that represent different *L. monocytogenes* strains in water and HI. The solid line is the regression fit of the data described by: $HI = 516.5 \times \text{adhesion energy (fJ)} + 13.2$, $r^2 = 0.91$. B) The relationship between *L. monocytogenes* virulence represented by the 50% lethal dose (LD50) and HI. The solid line is the regression fit of the data described by: $HI = -5.2 \times \ln(\text{virulence (LD50)}) + 187.5$, $r^2 = 0.88$. The linear and logarithmic relationships described above were chosen for simplicity.

Figure 7. Black and gray filled histograms showing the distribution of femto-Joule adhesion energy affinities measured successively between non-pathogenic and pathogenic *Listeria* surface biopolymers and silicon nitride in water. Solid lines indicate the log-normal peak function fits to the distribution of adhesion energies.

Table 1

A summary of all *Listeria* strains investigated in this study, the mean of their adhesion energies to silicon nitride in water ($\bar{E}$), the standard deviation of all adhesion energy measurements per strain (σ), the standard error of mean of all adhesion energy measurements per strain (σ_M) and HI values based on the standard error of mean. Shadowed rows indicate pathogenic *Listeria* strains while white-filled rows indicate non-pathogenic strains. For the non-pathogenic strains, there are no LD50 values because these strains are incapable of killing mice.

Strain	Serotype	Source	LD50 ⁵⁵	$\bar{E}$ (fJ)	σ (fJ)	n*	N*	σ_M (fJ)	HI
<i>L. monocytogenes</i> EGDe	1/2 a	Guinea pig	1.1×10 ⁷	0.168	0.064	11	165	0.0050	100
<i>L. monocytogenes</i> 874	Unknown	Cow brain	8.0×10 ⁷	0.154	0.054	10	150	0.0044	88.4
<i>L. monocytogenes</i> 1002	Unknown	Pork Sausage	5.2×10 ⁸	0.138	0.052	11	165	0.0041	81.1
<i>L. monocytogenes</i> 19115	4b	Human	6.0×10 ⁸	0.118	0.054	11	165	0.0042	84.2
<i>L. monocytogenes</i> 19112	2	Human	1.6×10 ⁹	0.130	0.058	14	210	0.0040	80.3
<i>L. monocytogenes</i> 19118	4e	Chicken	7.8×10 ⁹	0.122	.048	10	150	0.0039	77.8
<i>L. ivanovii</i> LMB1380	Unknown	Aborted calf	Unknown	0.109	0.039	17	255	0.0025	49.2
<i>L. innocua</i> 415	Unknown	Turkey burger		0.039	0.024	15	225	0.0016	32.4
<i>L. welshimeri</i> LMB0082	Unknown	Chicken		0.048	0.039	15	225	0.0026	52.5
<i>L. monocytogenes</i> 15313	1	Rabbit		0.070	0.044	26	390	0.0023	45.1
<i>L. seeligeri</i> RM308	Unknown	Soil		0.097	0.042	15	225	0.0028	55.6
<i>L. grayi</i> 700545	Unknown	Unknown		0.120	0.061	14	210	0.0038	76.9

* N is the number of curves and n is the number of cells used per strain