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MINIREVIEWS

Statistical Analysis of Long- and Short-Range Forces Involved in Bacterial Adhesion to Substratum Surfaces as Measured Using Atomic Force Microscopy▽

Yun Chen, Henk J. Busscher, Henny C. van der Mei,* and Willem Norde

W. J. Kolff Institute, Department of Biomedical Engineering, University Medical Center Groningen and University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Surface thermodynamic analyses of microbial adhesion using measured contact angles on solid substrata and microbial cell surfaces are widely employed to determine the nature of the adhesion forces, i.e., the interplay between Lifshitz-van der Waals and acid-base forces. While surface thermodynamic analyses are often viewed critically, atomic force microscopy (AFM) can also provide information on the nature of the adhesion forces by means of Poisson analysis of the measured forces. This review first presents a description of Poisson analysis and its underlying assumptions. The data available from the literature for different combinations of bacterial strains and substrata are then summarized, leading to the conclusion that bacterial adhesion to surfaces is generally dominated by short-range, attractive acid-base interactions, in combination with long-range, weaker Lifshitz-van der Waals forces. This is in line with the findings of surface thermodynamic analyses of bacterial adhesion. Comparison with single-molecule ligand-receptor forces from the literature suggests that the short-range-force contribution from Poisson analysis involves a discrete adhesive bacterial cell surface site rather than a single molecular force. The adhesion force arising from these cell surface sites and the number of sites available may differ from strain to strain. Force spectroscopy, however, involves the tedious task of identifying the minor peaks in the AFM retraction force-distance curve. This step can be avoided by carrying out Poisson analysis on the work of adhesion, which can also be derived from retraction force-distance curves. This newly proposed way of performing Poisson analysis confirms that multiple molecular bonds, rather than a single molecular bond, contribute to a discrete adhesive bacterial cell surface site.

Bacteria can adhere to various natural (33) and synthetic (11) surfaces, a phenomenon with widely different fields of application ranging from marine fouling, soil remediation, and food and drinking water processing to medicine and dentistry. In order to avoid the problems sometimes associated with bacterial adhesion, or to take advantage of it, better understanding of the mechanisms by which bacteria adhere to surfaces is required.

Bacterial adhesion to surfaces can be approached by biochemical methods, by which the molecular structures mediating adhesion are unraveled (5, 18, 22, 31), or by physicochemical methods. Surface thermodynamic analyses of bacterial cell and substratum surfaces using measured contact angles with liquids have not only indicated when thermodynamic conditions are favorable or unfavorable for adhesion (1, 37) but can also be employed in combination with measured zeta potentials of the interacting surfaces to determine the nature of the adhesion forces that mediate initial adhesion, i.e., the interplay among long-range (Lifshitz-van der Waals [LW] and electrical double-layer [EDL]) and short-range (Lewis acid-base [AB]) interaction forces (35). Surface thermodynamic analyses of bacterial adhesion have always been questioned, however, due to the macroscopic nature of the approach, among other concerns (10, 14, 15, 17, 32).

While surface thermodynamic analyses are often viewed critically, atomic force microscopy (AFM) can also provide information on the nature of bacterial adhesion forces by means of Poisson analysis of the measured forces. AFM spectroscopy reveals the distance dependence of the adhesion force, and measured force-distance curves can be compared with theoretical models (3, 7, 8, 13), which are usually based on the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (12, 39). The extended DLVO theory (36) includes not only long-range LW and EDL interactions, as does the classical DLVO theory, but also short-range AB interactions. The total interaction force between a bacterium and a substratum surface can be assigned to a variety of individual single bonds, but direct measurement of the single-bond forces requires high AFM resolution (19), which is often not available on commercial AFM instruments. As an alternative, a statistical method, called Poisson analysis, was first applied by Han, Williams, and Beebe (19, 43) to determine the magnitude of individual LW bonds between an AFM tip and a gold surface, as well as the strength of an individual AB bond between an AFM tip and a mica surface. Soon afterwards, Poisson analysis of AFM adhe-

* Corresponding author. Mailing address: W. J. Kolff Institute, Department of Biomedical Engineering, University Medical Center Groningen and University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. Phone: 31-50-3633140. Fax: 31-50-3633159. E-mail: h.c.van.der.mei@med.umcg.nl.

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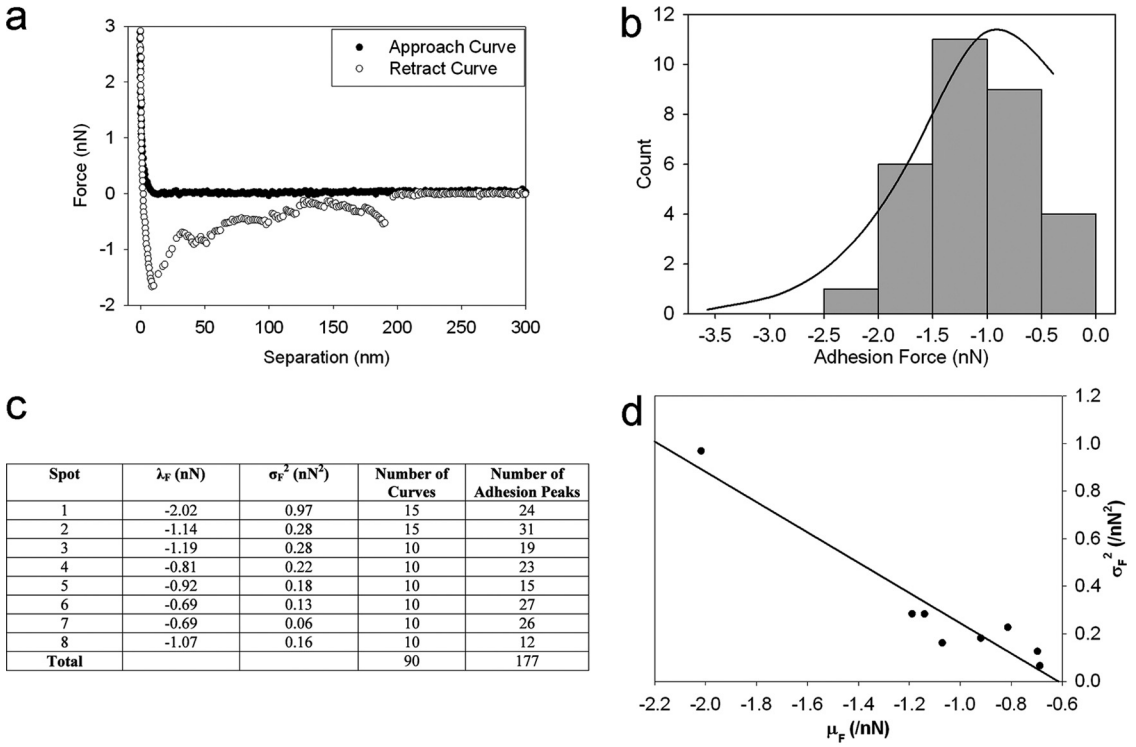


Spot	λ_F (nN)	σ_F^2 (nN^2)	Number of Curves	Number of Adhesion Peaks
1	-2.02	0.97	15	24
2	-1.14	0.28	15	31
3	-1.19	0.28	10	19
4	-0.81	0.22	10	23
5	-0.92	0.18	10	15
6	-0.69	0.13	10	27
7	-0.69	0.06	10	26
8	-1.07	0.16	10	12
Total			90	177

FIG. 1. Example of the steps involved in Poisson analysis of bacterial adhesion forces measured using AFM for a single bacterial (*S. epidermidis* ATCC 35983) probe interacting with eight different spots on a glass surface, with at least 10 force-distance curves taken at each spot. (a) Example of an AFM retraction curve including multiple adhesion peaks. (b) Histogram of adhesion forces at a single spot. The solid line indicates the fit of the data to a Poisson distribution. (c) Average adhesion force (λ_F) and its variance (σ_F^2) over the number of adhesion peaks from all force-distance curves taken at one spot. (d) σ_F^2 as a function of λ_F, yielding a straight line according to equation 6, from which the single-bond short-range force (f_{SR}) (-0.64 ± 0.08 nN) and the long-range force (F_{LR}) (-0.62 ± 0.14 nN) can be calculated (R^2, 0.916 for the example given).

sion forces was used to determine the nature of the interaction forces between single molecules (25, 34, 41, 42) and the nature of the forces mediating bacterial adhesion to surfaces (2, 5, 8, 10, 26, 27).

This review first describes the principles and underlying assumptions of Poisson analysis of adhesion forces measured by AFM and then summarizes the bacterial-adhesion data available in the literature. Finally, it suggests that the assumptions underlying Poisson analysis of adhesion forces obtained by use of AFM may be better met by analyzing the work of adhesion, which can be derived from retraction force-distance curves measured by AFM, than by analyzing adhesion forces.

THEORETICAL BACKGROUND OF POISSON ANALYSIS OF ADHESION FORCES

The adhesion force between two surfaces may be considered the sum of a finite number of discrete single bonds (20, 29). Assuming that the formation of a single bond is random and that all bonds develop independently with similar strengths (19, 43), the number of bonds should follow a binomial distribution (19). The adhesion force is composed of long-range LW and EDL forces and short-range AB forces. The long-range forces decay relatively slowly with distance and can be considered constant at close range. Moreover, at close range, the magnitude of long-range forces is generally small compared to that of short-range forces (38). Consequently, at a relatively short separation distance, the variation of the total adhesion force (F) is due mainly to variations in the occurrence of the short-range forces. F can accordingly be expressed as follows:

$$F = (k \times f_{SR}) + F_{LR} \quad (1)$$

where k is the number of short-range bonds, f_{SR} is the magnitude of a single short-range bond, and F_{LR} represents the long-range-force contribution to the adhesion force.

Figure 1a shows an AFM force-distance curve for *Staphylococcus epidermidis* on glass with multiple peaks in the retraction curve. Considering each adhesion peak as an individual detachment event (2, 5, 8, 10, 26, 27), each peak provides a specific adhesion force (F) according to equation 1, and the only variable for a given combination of bacterial strain and substratum is the number of short-range bonds (k). It should be noted that it is a tedious task to identify the minor peaks, since it is not clear *a priori* when a peak should be taken as an individual detachment event.

The distribution of k is reflected in the distribution of the adhesion force (F) over multiple adhesion peaks from a group of repeated AFM measurements over the same bacterial cell surface (Fig. 1b). The distribution of F should approximately follow a Poisson distribution, provided the sample size is sufficiently large, and each bond forms with a small probability (2), according to equation 2:

TABLE 1. Short-range and long-range force contributions to the adhesion of different bacterial strains to substratum surfaces[a]

Bacterial strain and substratum	Adhesion force component (nN)		Reference
	f_{SR}	F_{LR}	
Escherichia coli JM109 vs Si_3N_4	−0.125	−0.155[b]	2
Pseudomonas aeruginosa PAO1 vs BSA-coated glass	−0.31	−0.03[c]	5
Pseudomonas aeruginosa AK1401 vs BSA-coated glass	−0.44	−0.09[c]	
Staphylococcus epidermidis 3399 vs glass	−0.24	−0.07[b]	8
Staphylococcus epidermidis ATCC 35983 vs glass	−0.79	−0.33[b]	
Staphylococcus epidermidis HBH_2 3 vs glass	−1.02	−0.58[b]	
Staphylococcus epidermidis HBH_2 169 vs glass	−0.75	−0.41[b]	
Streptococcus mitis BMS vs saliva-coated enamel	−1.0 ± 0.2	−0.3 ± 0.1[b]	27
Streptococcus sanguinis ATCC 10556 vs saliva-coated enamel	−1.1 ± 0.2	−0.3 ± 0.1[b]	
Streptococcus sobrinus HG1025 vs saliva-coated enamel	−0.8 ± 0.1	−0.3 ± 0.1[b]	
Streptococcus mutans ATCC 700610 vs saliva-coated enamel	−0.8 ± 0.2	−0.4 ± 0.1[b]	
Streptococcus sanguinis ATCC 10556 vs stainless steel	−0.6 ± 0.2	8.1 ± 2.1[b]	26
Streptococcus mutans ATCC 700610 vs stainless steel	−0.5 ± 0.1	1.2 ± 0.3[b]	
Streptococcus sanguinis ATCC 10556 vs saliva-coated stainless steel	−0.9 ± 0.2	−0.3 ± 0.1[b]	
Streptococcus mutans ATCC 700610 vs saliva-coated stainless steel	−0.7 ± 0.1	−0.5 ± 0.2[b]	

[a] Adhesion of bacteria to saliva-coated surfaces may involve only a few ligand-receptor interactions.
[b] F_{LR} values from the original publication are corrected, because of an erroneous sign for the force values published.
[c] F_{LR} values were not presented in the original publication but have been calculated based on supplementary data for the original publication (5).

$$P(F, \lambda_k) = \frac{\lambda_k \times e^{-\lambda_k}}{k!} \quad (2)$$

where k is the bond number corresponding to the adhesion force (F), and $P(F, \lambda_k)$ is the probability for a specific value of F. λ_k is the population mean of k, according to equation 3:

$$\lambda_k = \sum_{k=0}^{+\infty} [k \times P(F, \lambda_k)] \quad (3)$$

One unique feature of a Poisson distribution is that its variance is always equal to the population mean, i.e.,

$$\sigma_k^2 = \lambda_k \quad (4)$$

Thus, the population mean and variance of the adhesion force (λ_F and σ_F^2) can be expressed as follows:

$$\lambda_F = (\lambda_k \times f_{SR}) + F_{LR} \quad (5)$$

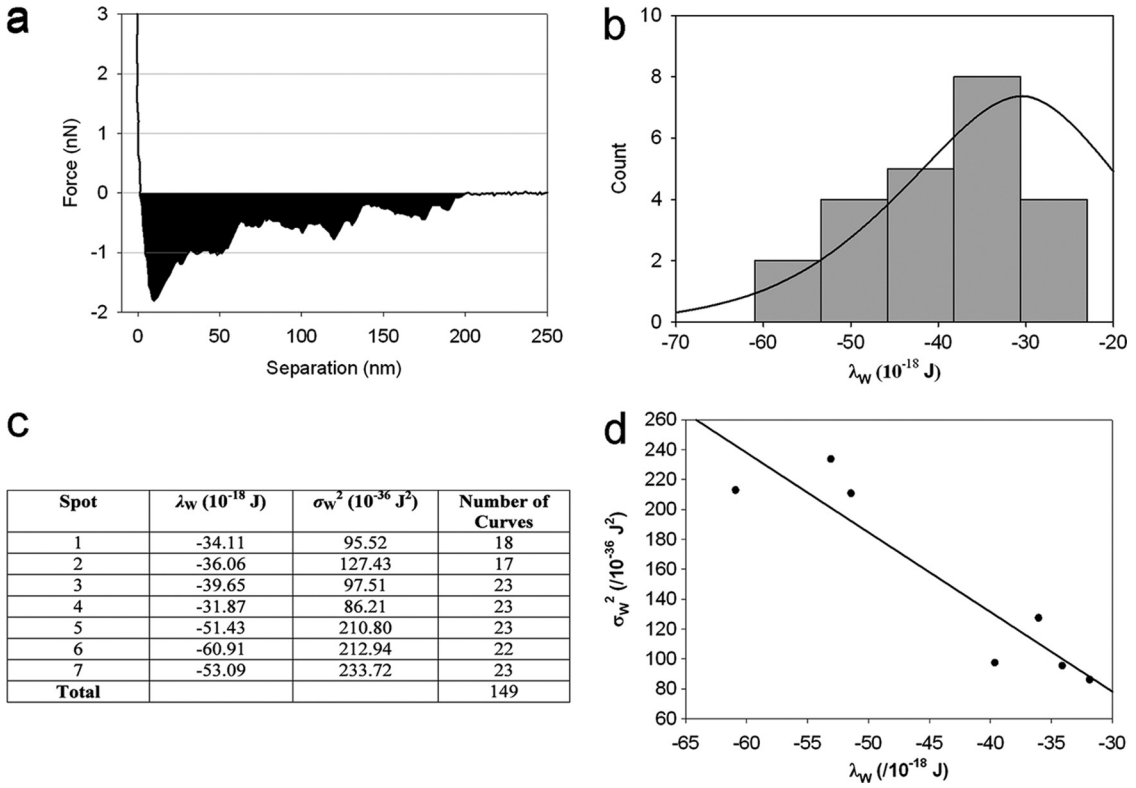
$$\sigma_F^2 = \sigma_k^2 \times f_{SR}^2 = \lambda_k \times f_{SR}^2 = f_{SR} \times (\lambda_F - F_{LR})$$
$$= f_{SR} \times \lambda_F - f_{SR} \times F_{LR} \quad (6)$$

In statistics, the population mean and variance are often inaccessible and can only be estimated from sufficiently large sample sizes (6), e.g., over a series of force-distance curves measured for the same bacterium. Figure 1c lists the λ_F and σ_F^2 values calculated for a single bacterial probe interacting with eight different spots on a glass surface, with at least 10 force-distance curves taken at each spot. From equation 6 it follows that σ_F^2 relates linearly with λ_F, as illustrated in Fig. 1d. The slope of the line represents the magnitude of f_{SR}, while its intercept equals $(-f_{SR} \times F_{LR})$, from which the long-range-force contribution (F_{LR}) can be derived.

POISSON ANALYSIS OF BACTERIAL ADHESION FORCES

Table 1 summarizes the results of Poisson analyses of bacterial adhesion forces obtained from AFM that, to our knowledge, have been published to date. A negative force value indicates attraction, while a positive value stands for repulsion. The short-range single-bond force values (f_{SR}) are always negative and vary by almost a factor of 10 among the different combinations of strains and substrata. Note that f_{SR} is the strength of an individual short-range bond, and not the total short-range force, which equals the number of short-range bonds (k) times the individual short-range-bond strength (f_{SR}) (see equation 1). The number of short-range bonds can vary widely, and Poisson analysis indicated that about 12 bonds were formed between an *Escherichia coli* strain and a silicon nitride AFM tip (2), while a single streptococcus attached to stainless steel through 60 short-range bonds (26), a number that decreased to 3 or even fewer when the steel surface was coated with saliva (27). This implies that only a very few ligand-receptor bonds are involved in the adhesion of streptococci to saliva-coated surfaces, making it doubtful whether the formation of ligand-receptor bonds should be considered a random event, as is required in Poisson analysis. A similarly low number of single short-range bonds can be inferred for *Pseudomonas aeruginosa* adhering to a bovine serum albumin (BSA) coating (5) and for staphylococci adhering to glass (8).

Poisson analysis directly yields the total long-range force (F_{LR}) (Table 1). The long-range forces are attractive for all combinations of bacterial strains and substrata, except for streptococci on conducting substrata, on which the long-range forces appear repulsive due to additional EDL repulsion between negatively charged bacteria and negative image charges in the stainless steel. For nonconducting substrata, the short-range single-bond force is comparable to, or even stronger than, the total long-range-force contribution, indicating that the total short-range force exceeds the long-range force (F_{LR}). This supports the general interpretation that the short-range single-bond force (f_{SR}) derived from Poisson analysis is due to AB forces, while LW and EDL forces contribute to the long-range force (F_{LR}), which is in line with most surface thermodynamic analyses of bacterial adhesion and with extended DLVO analyses (7, 9, 23, 28, 30).



Spot	λ_W (10^{-18} J)	σ_W^2 (10^{-36} J^2)	Number of Curves
1	-34.11	95.52	18
2	-36.06	127.43	17
3	-39.65	97.51	23
4	-31.87	86.21	23
5	-51.43	210.80	23
6	-60.91	212.94	22
7	-53.09	233.72	23
Total			149

FIG. 2. Example of the proposed Poisson analysis of the work of adhesion, calculated from retraction force-distance curves measured by AFM for *S. epidermidis* ATCC 35983 interacting with glass. (a) Example of an AFM retraction curve from which the work of adhesion (W_{adh}; black area) is calculated. (b) Histogram of different values for the work of adhesion at a single spot. The solid line indicates the fit of the data to a Poisson distribution. (c) Average work of adhesion (λ_W) and its variance (σ_W^2) over all force-distance curves taken at one spot. (d) σ_W^2 as a function of λ_W, yielding a straight line according to equation 8, from which the single-bond short-range contribution (w_{SR}) [$(-5.3 \pm 1.0) \times 10^{-18}$ J] and the long-range contribution (W_{LR}) [$(-15.3 \pm 8.3) \times 10^{-18}$ J] to the work of adhesion can be calculated (R^2, 0.854 for the example given).

Although the single-bond forces derived from Poisson analysis are widely interpreted as hydrogen bonds (5, 8, 26, 27), the typical rupture forces of a hydrogen bond are reportedly about 0.01 nN (19, 20), orders of magnitude smaller than the f_{SR} values in Table 1. On the other hand, ligand-receptor bonds, e.g., the streptavidin-biotin interaction, are reported to be on the order of 0.1 nN (4, 16, 24, 25), which is comparable to the f_{SR} values in Table 1 and suggests that multiple hydrogen bonds are involved in one ligand-receptor bond. Indeed, X-ray crystallography has proven that multiple hydrogen bonds are involved in ligand-receptor bonds (40). Consequently, one can conclude that the short-range-force contribution from Poisson analysis involves a discrete adhesive bacterial cell surface site rather than a single molecular force. The adhesion force arising from these cell surface sites and the number of sites available may differ from strain to strain (Table 1).

POISSON ANALYSIS OF THE WORK OF BACTERIAL ADHESION

Hitherto, Poisson analysis of bacterial adhesion data obtained using AFM has always been based on adhesion forces (2, 5, 8, 10, 25, 26, 34). However, considering the difficulties involved in the identification of minor peaks in the retraction force-distance curves, a much simpler and less bias prone method might be to perform the analysis on the basis of the work of adhesion (W_{adh}), which represents the free energy required to detach a bacterium from a substratum surface and can be calculated from the area under the entire retraction force-distance curve. In analogy to equation 1, the work of adhesion can be expressed as follows:

$$W_{adh} = (n \times w_{SR}) + W_{LR} \quad (7)$$

where w_{SR} and W_{LR} are the short-range single-bond and long-range contributions to the work of adhesion, respectively. By following the deduction process described for equation 6, it can be shown that

$$\sigma_w^2 = w_{SR} \times (\lambda_w - W_{LR}) \quad (8)$$

which yields w_{SR} and W_{LR} from linear regression of a plot of σ_W^2 versus λ_W, as demonstrated in Fig. 2d for *S. epidermidis* ATCC 35983 interacting with a glass surface. Both short-range and long-range contributions indicate favorable conditions for adhesion. The long-range energy (W_{LR}) is generally a couple of orders of magnitude larger than literature values based on surface thermodynamic and (extended) DLVO analyses (7, 9, 23, 28, 30), while the attractive AB interaction energies from the literature are comparable to the total short-range contribution derived here. A comparison of the w_{SR} values resulting from the newly proposed analysis with hydrogen-bonding energies (10 to 40 kJ/mol) from the literature (21) confirms our

conclusion based on force analysis that Poisson analysis involves a discrete adhesive bacterial cell surface site, composed of multiple AB bonds, rather than a single molecular bond.

Poisson analysis of the work of adhesion has a statistical drawback in that one retraction force-distance curve yields only a single value for the work of adhesion (Fig. 2a), whereas in the force analysis, multiple peaks from a single retraction force-distance curve are involved, as shown in Fig. 1c. As a consequence, the sample size required for use in Poisson analysis must be increased, although care must be taken to minimize possible damage to the bacterial cell surface. In our experience, however, 20 measurements at each spot can provide a sufficient number of data for Poisson analysis of the work of adhesion without significant damage to the bacterial cell surface (Fig. 2c).

The assumptions necessary for the Poisson analysis of adhesion forces are mostly valid for the Poisson analysis of the work of adhesion. However, in the case of force analysis, the long-range force (F_{LR}) is assumed to be constant among different individual adhesion peaks, since each peak is considered to represent an individual detachment event. Yet it is known that long-range forces, such as LW interactions, also decay with distance and will have a smaller magnitude in more-distant minor peaks. For Poisson analysis of the work of adhesion, one retraction force-distance curve represents complete bacterial cell detachment as an independent detachment event, and accordingly, the long-range work of adhesion can be considered invariant among all retraction force-distance curves.

CONCLUSIONS

Poisson analysis of bacterial adhesion forces measured by AFM provides a means of determining the short-range and long-range contributions to the force of adhesion. The resulting short-range-force contributions could be interpreted in line with the AB interactions from surface thermodynamic and (extended) DLVO analyses of bacterial adhesion, while long-range forces are significantly larger than the literature values for the LW and EDL forces. However, contrary to what would be expected from this type of analysis, the short-range-force contribution from Poisson analysis involves a discrete adhesive bacterial cell surface site rather than a single molecular force. The adhesion force arising from these cell surface sites and the number of sites available may differ from strain to strain. Moreover, it is unlikely that all cell surface sites have equal strength, but currently there are no methods to demonstrate this experimentally. It is possible, though, that the assumption of equal strength reduces the variance (σ_F^2), thereby affecting the decoupling of adhesion forces according to equation 6.

An alternative to carrying out Poisson analysis on the force of adhesion would be to perform Poisson analysis on the work of adhesion, which is also derived from retraction force-distance curves obtained using AFM. This alternative obviates the tedious identification of minor peaks in the AFM retraction force-distance curves. Poisson analysis of the work of adhesion confirms that the analysis involves a discrete adhesive bacterial cell surface site, composed of multiple AB bonds, rather than a single molecular bond.

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