Quantitatively Predicting Bacterial Adhesion Using Surface Free Energy Determined with a Spectrophotometric Method

Xinru Zhang,^{†,‡} Qian Zhang,[§] Tao Yan,[§] Zeyi Jiang,^{||} Xinxin Zhang,^{||} and Yi Y. Zuo^{*,†,⊥}

[†]Department of Mechanical Engineering, and [§]Department of Civil and Environmental Engineering, University of Hawaii at Manoa, Honolulu, Hawaii 96822, United States

[‡]School of Materials Science and Engineering, and ^{||}School of Mechanical Engineering, University of Science and Technology Beijing, Beijing 100083, People's Republic of China

[⊥]Department of Pediatrics, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96826, United States

S Supporting Information

ABSTRACT: Bacterial adhesion onto solid surfaces is of importance in a wide spectrum of problems, including environmental microbiology, biomedical research, and various industrial applications. Despite many research efforts, present thermodynamic models that rely on the evaluation of the adhesion energy are often elusive in predicting the bacterial adhesion behavior. Here, we developed a new spectrophotometric method to determine the surface free energy (SFE) of bacterial cells. The adhesion behaviors of five bacterial species, *Pseudomonas putida* KT2440, *Salmonella* Typhimurium ATCC 14028, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* DH5 α , onto two model substratum surfaces, i.e., clean glass and silanized glass surfaces, were studied. We found that bacterial adhesion was unambiguously mediated by the SFE difference between the bacterial cells and the solid substratum. The lower the SFE difference, the higher degree of bacterial adhesion. We therefore propose the use of the SFE difference as an accurate and simple thermodynamic measure for quantitatively predicting bacterial adhesion. The methodological advance and thermodynamic simplification in the paper have implications in controlling bacterial adhesion and biofilm formation on solid surfaces.

INTRODUCTION

Bacterial adhesion onto solid surfaces is the onset of biofilm formation, which plays a central role in a wide spectrum of problems, including environmental microbiology, biomedical research, and various industrial applications.^{1–7} On the one hand, bacterial adhesion is commonly regarded as the villain in a series of adverse environmental and health issues, such as biofouling, biocorrosion, and infection of implant biomaterials and the respiratory system.^{8–11} On the other hand, bacterial adhesion can be used to play a beneficial role in bioremediation of wastewater, microbial fuel cells,¹² and the mineral beneficiation process.^{6,13} Therefore, understanding and controlling bacterial adhesion are of significant scientific and engineering interests.

Adhesion of bacteria to solid surfaces is a complex process affected by multiple factors.^{3,7,14} First, bacterial adhesion is influenced by physicochemical properties of the bacterial cells, such as their surface free energy (SFE),^{15–17} ζ potential,^{18,19} production of extracellular polymeric substances, and the presence of pili and flagella.^{2,20,21} Second, bacterial adhesion is affected by the surface properties of substratum surfaces, including their hydrophobicity,^{8,18,22–24} ζ potential,^{2,7,18} roughness, and microtopography.^{2,25–28} Third, bacterial adhesion can be also mediated by the suspending liquid medium, including its surface tension,²⁹ pH,³⁰ ionic strength,³¹ temperature, and

hydrodynamics.^{7,32,33} All of these factors affect the onset and progression of bacterial adhesion to solid surfaces.

Among all of these influencing factors, the SFEs of bacterial cells and solid surfaces have long been known to play a major role in bacterial adhesion. Neumann and co-workers developed a pioneering surface thermodynamic model to predict bacterial adhesion by evaluating the thermodynamic adhesion energy (ΔF_{adh}) between the bacterial cells and solid surfaces.²⁹ In addition, bacterial adhesion has been studied within the scope of the classical Derjaguin–Landau–Verwey–Overbeek (DLVO)^{3,14} and the extended DLVO (xDLVO)^{4,13,34} theories, in which the adhesion energy between bacteria and surfaces is interpreted with intermolecular and surface forces, including the van der Waals forces, electrostatic interactions, and near-surface Lewis acid–base interactions.³⁵

Regardless of these theoretical and experimental efforts, the existing thermodynamic models are often elusive in predicting bacterial adhesion.^{3,20,36–38} This is in part due to the difficulty in accurately determining the SFE of bacterial cells, which is a central parameter needed in all thermodynamic models of

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bacterial adhesion. In comparison to the SFE of solid substratum surfaces, it is technically challenging to determine the SFE of bacterial cells. A common way of measuring the SFE of live cells is to first prepare a cell layer/lawn followed by Young's contact angle measurement and interpretation on this cell layer as if it was a regular ideal solid surface.^{15,18,39,40} The cell layer is routinely prepared by filtration, in which it is difficult to control the compactness, roughness, thickness, and even the number of filtrated cell layers. Plenty of experimental evidence showed that the contact angle measured on filtrated cell layers varies with the volume of the sessile drop, thickness, and drying time of the cell layers.^{15–17,39} All of these technical difficulties introduce significant methodological complication and uncertainty in determining the SFE of live cells and, hence, compromise the thermodynamic analysis of bacterial adhesion.

We recently developed a novel spectrophotometric method for directly determining the SFE of live cells.^{41,42} This method relies on DLVO analysis of colloidal stability and is implemented by simple spectrophotometric measurements. It is accurate, inexpensive, easy-to-use, and high-throughput. We have demonstrated its feasibility in determining the SFEs of abiotic particles, microalgae, and bacterial cells.^{41,42} In this paper, we revisit the classical surface thermodynamic model for bacterial adhesion,²⁹ using the bacterial SFE determined by our new spectrophotometric method. We hypothesize that by accurately determining the bacterial SFE, the adhesion behavior of bacterial cells onto model solid surfaces can be quantitatively predicted by the difference in SFE between the bacterial cells and the solid surfaces.

EXPERIMENTAL SECTION

Thermodynamic Adhesion Energy. Similar to the prediction of liquid spreading on a solid surface by the Dupré equation, it is generally accepted that bacterial adhesion can be qualitatively predicted by the thermodynamic adhesion energy (ΔF_{adh}).²⁹ A negative ΔF_{adh} leads to bacterial adhesion, whereas a positive ΔF_{adh} does not

$$\Delta F_{\text{adh}} = \gamma^{\text{bs}} - \gamma^{\text{bl}} - \gamma^{\text{sl}} \quad (1)$$

where γ^{bs} , γ^{bl} , and γ^{sl} are the interfacial free energies of the bacteria–solid, bacteria–liquid, and solid–liquid interfaces.

These interfacial free energies (γ^{bs} , γ^{bl} , and γ^{sl}) can be calculated from the corresponding SFEs (γ^{bv} , γ^{sv} , and γ^{lv}) via appropriate combining rules.^{39,40} Without the loss of generality, using Neumann's equation of state,⁴³ γ^{bs} , γ^{bl} , and γ^{sl} can be expressed as

$$\gamma^{\text{bs}} = \gamma^{\text{bv}} + \gamma^{\text{sv}} - 2\sqrt{\gamma^{\text{bv}}\gamma^{\text{sv}}}e^{-\beta(\gamma^{\text{bv}}-\gamma^{\text{sv}})^2} \quad (2)$$

$$\gamma^{\text{bl}} = \gamma^{\text{bv}} + \gamma^{\text{lv}} - 2\sqrt{\gamma^{\text{bv}}\gamma^{\text{lv}}}e^{-\beta(\gamma^{\text{bv}}-\gamma^{\text{lv}})^2} \quad (3)$$

$$\gamma^{\text{sl}} = \gamma^{\text{sv}} + \gamma^{\text{lv}} - 2\sqrt{\gamma^{\text{sv}}\gamma^{\text{lv}}}e^{-\beta(\gamma^{\text{sv}}-\gamma^{\text{lv}})^2} \quad (4)$$

where the value of β was experimentally determined to be $0.0001247 \text{ (mJ/m}^2\text{)}^{-2}$.⁴³

Substituting eqs 2–4 into eq 1 yields

$$\begin{aligned} \Delta F_{\text{adh}} = & 2\sqrt{\gamma^{\text{bv}}\gamma^{\text{lv}}}e^{-\beta(\gamma^{\text{bv}}-\gamma^{\text{lv}})^2} + 2\sqrt{\gamma^{\text{sv}}\gamma^{\text{lv}}}e^{-\beta(\gamma^{\text{sv}}-\gamma^{\text{lv}})^2} \\ & - 2\sqrt{\gamma^{\text{bv}}\gamma^{\text{sv}}}e^{-\beta(\gamma^{\text{bv}}-\gamma^{\text{sv}})^2} - 2\gamma^{\text{lv}} \end{aligned} \quad (5)$$

With eq 5, the adhesion energy (ΔF_{adh}) can be estimated by measuring SFEs of the bacterial cells (γ^{bv}), the solid substratum

(γ^{sv}), and the liquid medium (γ^{lv}) (note that the superscript v represents the vapor phase in which the SFE is determined). It can be derived from eq 5 that, within the same liquid medium (i.e., constant γ^{lv}), the smaller the SFE difference between the bacterial cells and the solid surface (i.e., $|\gamma^{\text{bv}} - \gamma^{\text{sv}}|$), the more negative the adhesion energy and, hence, more bacterial adhesion is expected. In other words, the degree of bacterial adhesion should increase with diminishing energy difference between the bacterial cells and the substratum surface. We will test this hypothesis in the following experiments.

Bacteria Culture and Preparation of Substratum Surfaces. We focused on five widely studied bacterial species, i.e., *Pseudomonas putida* KT2440, *Salmonella* Typhimurium ATCC 14028, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* DHS α (see Table S1 of the Supporting Information for a summary of morphological and surface properties of these bacterial cells). All bacteria except *S. epidermidis* were cultured in Luria–Bertani (LB) broth. *S. epidermidis* was cultured in a nutrient broth consisting of 10 g of tryptone, 3 g of beef extract, and 5 g of NaCl in 1 L of water. All bacteria were harvested at the stationary phase (OD₆₀₀ of approximately 2). The cultivated bacteria suspensions were centrifuged at 3600g for 3 min to remove cell debris, followed by three rounds of centrifugation and washing with phosphate buffer solutions (PBS). The bacteria cells were then resuspended in fresh culture medium, followed by 1 min vortex and 1 min sonication, to form homogeneous cell suspensions at concentrations of approximately 10^{10} cells/mL (for determining the SFE) and 10^8 cells/mL (for studying bacterial adhesion).

Two types of solid surfaces were prepared as the model substratum surfaces. Thoroughly cleaned microscopy glass slides (Millicell EZ SLIDE, Millipore, Germany) were used as a representative hydrophilic surface. Silanized glass slides were used as a representative hydrophobic surface. For glass silanization, microscopy glass slides were first cleaned with acetone and dried under nitrogen flow. The cleaned glass slides were then placed in a glass Petri dish at 70 °C for 12 h to allow for reaction with the vapor of 1,1,1,3,3,3-hexamethyldisilazane (HMDS, SPI Supplies, West Chester, PA).

Determination of SFEs of Cultural Medium (γ^{lv}), Substratum Surface (γ^{sv}), and Bacterial Cells (γ^{bv}). γ^{lv} of the cultural medium was determined with the pendant drop method using axisymmetric drop shape analysis (ADSA).⁴⁴ γ^{lv} of the LB broth was measured to be $62.0 \pm 0.2 \text{ mJ/m}^2$. γ^{lv} of the culture medium for *S. epidermidis* was measured to be $57.8 \pm 0.2 \text{ mJ/m}^2$. γ^{sv} was determined with the low-rate dynamic contact angle method in conjunction with Neumann's equation of state.⁴³ A detailed description of the low-rate dynamic contact angle method and intermediate experimental results can be found in the Supporting Information. γ^{sv} of the clean glass surface and the silanized glass surface was determined to be 70 ± 1 and $38 \pm 1 \text{ mJ/m}^2$, respectively. These measurements are in good agreement with literature values.⁴⁵

γ^{bv} was determined using our new spectrophotometric method. A detailed description of the spectrophotometric method can be found elsewhere.^{41,42} Principles of this method are based on the analysis of colloidal stability of cell suspensions. According to the classical DLVO theory, the colloidal stability of a cell suspension depends upon the balance between van der Waals attraction and electrostatic repulsion. A minimum van der Waals attraction can be obtained by dispersing cells in a liquid medium of which surface tension

Figure 1. Schematic of the spectrophotometric method for determining the SFE of bacterial cells (γ^{bv}). First, bacterial cells were dispersed into liquid media with a range of surface tensions (γ^{lv}). Second, the cell suspensions were centrifuged to separate the supernatant from the sediment. Third, the series of supernatants were transferred to a 96 well microplate. The degree of cell dispersion in these supernatants was compared by measuring their OD. Finally, OD of these supernatants was plotted against γ^{lv} . The location of the maximum OD, at which $\gamma^{bv} = \gamma^{lv}$, was determined with polynomial fitting.

Figure 2. Determination of the SFE of five bacterial cell species using the spectrophotometric method: (a) *P. putida*, (b) *Salmonella Typhimurium*, (c) *S. epidermidis*, (d) *E. faecalis*, and (e) *E. coli*. For each bacterial species, three individual experimental runs are presented to show the repeatability. Locations of the OD peaks correspond to the SFEs of these bacterial cells.

is close to the SFE of the cells, at which the cells are expected to disperse in the medium without significant aggregation and sedimentation. Hence, when dispersing cells in a series of liquids with known surface tension values, surface tension of the liquid with the maximum level of cell dispersion should be equal to the SFE of the cells. After removal of large aggregates with appropriate centrifugation (described in detail below), the degree of cell dispersion in these liquids can be compared by measuring the optical density (OD) of the suspension. The highest degree of cell dispersion corresponds to a maximum OD. Consequently, the surface tension of the liquid in which the maximum OD appears should be close to the SFE of the cells.

Implementation of the spectrophotometric method is illustrated in Figure 1. A series of liquid media with surface

tensions ranging from 22 to 72 mJ/m², each at 0.5 mL, were prepared with binary mixtures of ethanol and water at predefined mixing ratios. Surface tensions of these liquids were quantified by the pendant drop method. A total of 10 μ L bacterial suspension with a concentration of approximately 10^{10} cells/mL was added to each liquid medium, followed by vortex mixing and leaving undisturbed for 20 min. These liquid media were subsequently centrifuged at 100g for 6 min to separate the supernatant from the sediment. This centrifugation speed was carefully selected to be high enough to spin down aggregated cells but low enough to keep individually dispersed cells in the supernatant. We found nearly no separation happening in the liquid medium of which surface tension is close to the SFE of the bacterial cells. In this liquid medium, cells were uniformly dispersed without significant aggregation. A total of 200 μ L of

supernatant of each liquid medium was then transferred to a 96 well microplate. OD at the wavelength of 600 nm (OD_{600}) was measured by a microplate reader (Epoch, BioTek, Winooski, VT). OD_{600} was plotted against surface tensions of the liquid media, and peaks were determined by the third-order polynomial fitting. All measurements were performed at least 3 times and averaged.

Bacterial Adhesion Experiments. Bacterial adhesion was studied using a glass cell analysis chamber (Millicell EZ SLIDE), with the clean microscopy glass or the silanized glass slide as the substratum. A total of 1 mL of bacterial cell suspension at a concentration of 10^8 cells/mL was added to each chamber and incubated at 37 °C for 2 h. After incubation, the substratum was removed from the chamber, gently rinsed with PBS to remove non-adherent bacteria, and air-dried. Cells adherent to the substratum were stained by methylene blue and enumerated by an optical microscope (BX51, Olympus, Japan). The number of adhered bacterial cells was analyzed using ImageJ [National Institutes of Health (NIH)]. All measurements were performed at least 3 times, and results were shown in mean $\pm$ standard deviation (SD).

RESULTS AND DISCUSSION

SFE of Bacterial Cells (γ^{bv}). Figure 2 shows the experimental results of measuring the SFE of five bacterial species, *P. putida* (a), *Salmonella* Typhimurium (b), *S. epidermidis* (c), *E. faecalis* (d), and *E. coli* (e), using the spectrophotometric method. For each bacterial species, three experimental runs are presented to show the repeatability of our measurements. It can be seen that OD_{600} measured from different experimental runs notably scatters. This is due to variations in the total number of cells when dispersing a small amount (10 μ L) of highly concentrated (10^{10} cells/mL) cell suspensions in each experimental run. Despite scattering between experimental runs, the peak OD values determined from different experimental runs collapse into the same surface tension range for each bacteria studied. Locations of these OD peaks, indicating the maximum degree of cell dispersion, correspond to the characteristic SFEs of these bacterial cells, i.e., 35.5 ± 0.5 , 49.5 ± 0.9 , 59.3 ± 0.9 , 64.5 ± 0.9 , and 65.1 ± 0.5 mJ/m², respectively. These results are consistent with our previous measurements, in which the characteristic SFEs of various bacterial species were used for cell sorting.⁴²

Because of the overabundance of bacterial species and strains, we can only conduct a limited direct comparison between our measurements and those reported by others. Using contact angle measurement in conjunction with Neumann's equation of state, Sharma and Rao reported the SFE of *E. faecalis* ATCC 29212 to be 64.45 mJ/m².³⁹ This is in excellent agreement with our spectrophotometric measurement of the same bacterial cells, i.e., 64.5 mJ/m². The SFE of *E. coli* of various strains was reported to be around 66–67 mJ/m²,^{15,29,39} which is also in good agreement with our measurements, i.e., 65.1 mJ/m².

To further verify the accuracy of our spectrophotometric method, we have determined the SFE of these bacterial cells by carefully performing the classical contact angle method.^{29,40} Experimental details and results can be found in the Supporting Information. We found an excellent agreement between these two methods with a general discrepancy of less than 1 mJ/m² for the measured SFEs of bacterial cells. However, in comparison to the contact angle method, which involves measuring the low-rate dynamic contact angle (see Figure S3 of the Supporting Information) and theoretical interpretation

using Neumann's equation of state (see Table S3 of the Supporting Information), the spectrophotometric method is much simpler, quicker, and less dependent upon the skills of the operator.

Bacterial Adhesion to Substratum Surfaces. Figure 3 shows the images of bacterial adhesion onto clean glass surfaces

Figure 3. Images of bacterial adhesion onto clean glass surfaces (a1–e1) and silanized glass surfaces (a2–e2). The five bacterial species are *P. putida* (a), *Salmonella* Typhimurium (b), *S. epidermidis* (c), *E. faecalis* (d), and *E. coli* (e). (f) Number of adhered bacterial cells per unit surface area in the above cases. It appears that the high-SFE bacteria cells, *E. faecalis* (d1) and *E. coli* (e1), preferentially adhere to the high-SFE clean glass surfaces, while the low-SFE bacterial cells, *P. putida* (a2) and *Salmonella* Typhimurium (b2), selectively adhere to the low-SFE silanized glass surfaces.

Figure 4. Bacterial adhesion correlated with (a) adhesion energy (ΔF_{adh}) and (b) SFE difference between γ^{bv} and γ^{sv} , i.e., $|\gamma^{bv} - \gamma^{sv}|$. Abbreviations used in panel b are P.p, *P. putida*; S.T, *Salmonella Typhimurium*; S.e, *S. epidermidis*; E.f, *E. faecalis*; and E.c, *E. coli*.

(left column) and onto silanized glass surfaces (right column). Figure 3f shows the number of adhered bacterial cells per unit surface area on these solid substrata. It appears that the high-SFE bacteria cells, *E. faecalis* (Figure 3d1) and *E. coli* (Figure 3e1), preferentially adhere to the high-SFE (i.e., hydrophilic) clean glass surfaces, while the low-SFE bacterial cells, *P. putida* (Figure 3a2) and *Salmonella Typhimurium* (Figure 3b2), preferentially adhere to the low-SFE (i.e., hydrophobic) silanized glass surfaces.

Surface Thermodynamics of Bacterial Adhesion. To better understand the surface thermodynamics of bacterial adhesion, we calculated the adhesion energy (ΔF_{adh}) using eq 5 based on our experimentally determined SFEs (γ^{bv} , γ^{sv} , and γ^{lv}). The sign of ΔF_{adh} has long been used as a qualitative criterion to predict bacterial adhesion: a negative ΔF_{adh} leads to bacterial adhesion, whereas a positive ΔF_{adh} does not.^{3,19,29,36} Figure 4a shows ΔF_{adh} calculated for all bacteria–surface pairs shown in Figure 3. Among all 10 bacteria–surface pairs studied, ΔF_{adh} is negative for *P. putida* (Figure 3a2) and *Salmonella Typhimurium* (Figure 3b2) on silanized glass surfaces and *S. epidermidis* (Figure 3c1), *E. faecalis* (Figure 3d1), and *E. coli* (Figure 3e1) on clean glass surfaces, indicating thermodynamic conditions favorable for bacterial adhesion. ΔF_{adh} is positive for the other five bacteria–surface pairs, indicating unfavorable conditions. It appears that the sign of ΔF_{adh} generally agrees with the behavior of bacterial adhesion (Figure 3f). However, when the absolute value of ΔF_{adh} is small (i.e., close to zero), such as in the case of *S. epidermidis* adhering to clean or silanized glass surfaces, the sign of ΔF_{adh} fails to predict the behavior of bacterial adhesion (Figure 3c1 versus Figure 3c2).

The sign of ΔF_{adh} can be only used to qualitatively predict the behavior of bacterial adhesion but not for quantitative analysis.^{1,3,36,37} By analyzing eq 5, we found that the quantity of ΔF_{adh} appears to correlate with the SFE difference between the bacterial cells and the solid substratum $|\gamma^{bv} - \gamma^{sv}|$. In Figure 4b, we plot the number of adhered bacterial cells as a function of the bacteria–substratum SFE difference. It is clear that, regardless of the specific bacterial species and substratum surfaces (hydrophilic or hydrophobic), the degree of bacterial adhesion nearly linearly increases with decreasing SFE difference, i.e., diminishing energy difference between the bacterial cells and the substratum surface. In comparison to the sign of the adhesion energy ΔF_{adh} commonly used in predicting bacterial adhesion, we hereby propose the use of the bacteria–substratum SFE difference $|\gamma^{bv} - \gamma^{sv}|$ to quantitatively predict bacterial adhesion.

Up to now, we have limited our discussion into the absolute value of $|\gamma^{bv} - \gamma^{sv}|$. As shown in Figure 4b, we found that $|\gamma^{bv} - \gamma^{sv}|$ can be used as a simple criterion for predicting adhesion of different bacteria onto a hydrophobic surface (guided by the solid line) or onto a hydrophilic surface (guided by the dashed line). Similarly, $|\gamma^{bv} - \gamma^{sv}|$ can be used to predict adhesion of one bacterial species onto either a hydrophobic or hydrophilic surface. However, Figure 4b also shows that, even with a similar $|\gamma^{bv} - \gamma^{sv}|$, the extent of different bacteria attaching to different surfaces can vary significantly. For example, although $|\gamma^{bv} - \gamma^{sv}|$ is close between *P. putida* at the silanized glass and *E. coli* at the unmodified glass, the extent of bacterial adhesion in these two cases varies by a factor more than 2. This indicates that the absolute value of $|\gamma^{bv} - \gamma^{sv}|$ cannot be the only factor in determining the general pattern of bacterial adhesion. Without detailed investigation, it is interesting to point out that, for all bacterial species studied on the silanized glass surface ($\gamma^{sv} = 38 \text{ mJ/m}^2$), *P. putida* ($\gamma^{bv} = 35.5 \text{ mJ/m}^2$) is the only species of which $\gamma^{bv} < \gamma^{sv}$, i.e., with a negative sign of $(\gamma^{bv} - \gamma^{sv})$. This may indicate that the sign of $(\gamma^{bv} - \gamma^{sv})$, in addition to its absolute value, is also meaningful in affecting bacterial adhesion.

Implications in Cell Surface Hydrophobicity. Bacterial adhesion is the initial step of biofilm formation on any biotic or abiotic surface.⁴⁶ The driving forces for the freely suspended planktonic organisms to attach to a solid surface appear to be complex and are affected by multiple factors. Numerous studies have demonstrated that the bacterial cell surface hydrophobicity (CSH) plays an important role in the initial attachment of bacteria to solid surfaces.^{46,47} Despite the intrinsic heterogeneity of cell surfaces, the bacterial CSH reflects the overall contribution of molecular and topographic structures to the hydrophobic properties of microbial surfaces. It is found that bacteria with higher CSH have a higher tendency to attach to hydrocarbons and adhere more to hydrophobic nonpolar surfaces.⁴⁷ A series of techniques have been developed to measure bacterial CSH. The most popular techniques rely on the measurement of microbial adhesion to hydrocarbons (MATH).⁴⁸ The MATH-like assays compare the preference of bacteria to the aqueous phase and to a hydrocarbon phase or hydrophobic ligands. Hence, MATH only measures the relative CSH. MATH results from the literature can be hardly compared directly because of the different test conditions used in different studies.⁴⁹ More recent studies use alternative measures to quantify CSH, such as the contact angle measured on bacterial cell lawns¹⁵ and interfacial rheology measured at bacteria-adsorbed oil–water interfaces.⁴⁹

In this study, we have demonstrated the use of SFE as a quantitative measure of CSH. We found increasing SFE of the five bacterial species used in this study, i.e., *P. putida* (35.5 mJ/m²) < *Salmonella* Typhimurium (49.5 mJ/m²) < *S. epidermidis* (59.3 mJ/m²) < *E. faecalis* (64.5 mJ/m²) < *E. coli* (65.1 mJ/m²), indicating a decrease in CSH of these bacteria. The ranking of CSH determined with the SFE in our study is in good agreement with the ranking determined by Rühls et al. using the MATH method and their interfacial tension measurements, i.e., CSH of *P. putida* > *Salmonella* Typhimurium > *E. coli*.⁴⁹ Therefore, the development of our easy-to-use spectrophotometric method not only provides a practical means of determining the SFE of live cells but also creates a novel method of quantitatively determining the microbial CSH.

Implications in Biofilm Formation. Despite the many years of research efforts, the underlying mechanism behind bacterial adhesion is unknown. Considerable discrepancy exists in the literature when attempting to generalize bacterial adhesion behavior from phenomenological measurements with individual bacteria and surfaces. On the one hand, as summarized in Donlan's seminal review,⁷ many researchers found that bacteria attach more rapidly to hydrophobic nonpolar surfaces, such as plastics, than to hydrophilic surfaces, such as glass or metals. On the other hand, extensive research evidence shows that bacteria (mainly those related to food-processing surfaces, such as *Listeria monocytogenes*) attach more to high-energy hydrophilic surfaces, including stainless steel and glass, than to hydrophobic polymeric surfaces.⁵⁰

Our present study suggests that the actual behavior of bacterial adhesion is governed by the hydrophobicity (or SFE) of not only the colonized solid surfaces but also the bacterial cells. For the solid surface, its apparent hydrophobicity is affected by both roughness and physicochemical properties, mainly the SFE. For an ideal surface (smooth and homogeneous), the SFE is a measure of the intrinsic surface hydrophobicity determined by the chemical structure and intermolecular forces of the surface. The lower the SFE, the more hydrophobic the surface. For real surfaces, however, roughness usually contributes to the apparent surface hydrophobicity. Interrelations between hydrophobicity and roughness in real surfaces complicate energetic interpretation of bacterial adhesion. Here, we circumvented this difficulty by studying bacterial adhesion onto two smooth model surfaces, i.e., unmodified and silanized glass surfaces. Their differences in surface hydrophobicity are purely due to their different SFEs determined with the low-rate dynamic contact angle method in conjunction with Neumann's equation of state approach, i.e., the SFE of 70 mJ/m² for unmodified glass versus 38 mJ/m² for silanized glass.

As for the selection of bacteria, not only do these bacterial species show a gradient of CSH (or SFE), but they are also representative for biofilm formation under various environmental and clinical conditions. Among the three Gram-negative bacterial species used here, *E. coli* is perhaps the most well-studied model organism in microbiology. *Salmonella* Typhimurium is a common pathogenic microbe found in foodborne biofilms. *P. putida* is a plant-associated bacterium often found in soil biofilms and most studied for bioremediation and wastewater treatment. The two Gram-positive bacteria used in this study, i.e., *S. epidermidis* and *E. faecalis*, are most studied for biofilm formation related to nosocomial infections. Both *S. epidermidis* and *E. faecalis* have been found in biofilms developed on indwelling medical devices, implants, and living

tissues, such as the central venous catheter, urinary catheter, prosthetic heart valve, and artificial voice prosthesis.⁵¹

After carefully determining SFEs of the model substratum surfaces and the bacterial cells, we found that the extent of bacterial adhesion can be quantitatively correlated with the SFE difference between the bacterial cells and the substrata, i.e., $|\gamma^{bv} - \gamma^{sv}|$. This finding is important because it suggests that bacterial adhesion, at least the initial attachment, is driven by a pure energetic potential. Instead of simply stating that bacteria prefer to attach to a hydrophobic or hydrophilic surface, our study provides a more general criterion for quantitatively predicting bacterial adhesion. We argue that bacteria prefer to attach to solid surfaces of similar SFE or hydrophobicity. In other words, the smaller the SFE difference between the bacteria and the solid surface $|\gamma^{bv} - \gamma^{sv}|$, the more adhesion is expected. It should be emphasized that feasibility of using the SFE difference as a practical criterion to evaluate bacterial adhesion stems from our novel high-throughput spectrophotometric method for determining the bacterial SFE, which can be easily operated in any lab with a microplate reader.

Cautions, however, should be exercised when generalizing our findings to biofilm formation because our experiments were conducted under strictly controlled laboratory conditions. Microbial colonization on real surfaces is much more complicated than on our model substrata because the apparent hydrophobicity of a real surface is affected by its roughness and heterogeneity. More importantly, the bacterial CSH has been shown to be affected by the microbial growth rate, growth medium, and culture conditions.⁵⁰ Hence, the bacterial CSH can change dramatically at different stages of cell dividing and in response to changes in the environment.⁴⁶ All of these factors may introduce deviations from our energetic interpretation of bacterial adhesion.

In summary, we have developed a novel high-throughput spectrophotometric method that allows us to accurately determine the SFE of live bacterial cells. Using this method, we have studied the adhesion behavior of five bacterial species onto two model substratum surfaces. We showed that the SFE difference between the bacterial cells and the solid substratum, i.e., $|\gamma^{bv} - \gamma^{sv}|$, can be used as an accurate and simple thermodynamic criterion for quantitatively predicting bacterial adhesion. The methodological advance and thermodynamic simplification have scientific and engineering implications in controlling bacterial adhesion and biofilm formation on solid surfaces.

■ ASSOCIATED CONTENT

Supporting Information

Summary of morphological and surface properties for bacterial cells used in this study (Table S1), schematic of the low-rate dynamic contact angle method (Figure S1), representative experimental results (i.e., contact angle, contact radius, and drop volume) for low-rate dynamic contact angle measurements on a clean glass surface (a) and a silanized glass surface (b) (Figure S2), results for determining γ^{sv} of clean glass and silanized glass surfaces (Table S2), representative experimental results (i.e., contact angle, contact radius, and drop volume) for low-rate dynamic contact angle measurements on carefully prepared bacteria lawns: (a) *P. putida*, (b) *Salmonella* Typhimurium, (c) *S. epidermidis*, (d) *E. faecalis*, and (e) *E. coli* (Figure S3), comparison of the SFE of bacterial cells (γ^{bv}) determined with the classical contact angle method and the spectrophotometric method (Table S3), and results for

determining the adhesion energy (ΔF_{adh}) of bacterial cells on the clean glass surface ($\gamma^{sv} = 70 \text{ mJ/m}^2$) and the silanized glass surfaces ($\gamma^{sv} = 38 \text{ mJ/m}^2$) (Table S4). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/es5050425.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 808-956-9650. Fax: 808-956-2373. E-mail: yzuo@hawaii.edu.

Notes

The authors declare no competing financial interest.

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