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Impact of Physiological State on Surface Thermodynamics and Adhesion of *Pseudomonas aeruginosa*

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A quantitative understanding of microorganism migration in geological formations is critical to predict the dissemination of microorganisms in the environment and to evaluate the efficacy of microbially mediated *in situ* pollutant degradation. The key event that retards the movement of microorganisms in the saturated zone with respect to the convective water flow is the interaction between microorganisms and the matrix surfaces. This interaction may result in adhesion and concomitant retardation. Interactions are determined by the surface thermodynamics of the microorganism and the matrix. Whereas the nature of the matrix substratum surface may be considered temporally invariant, the nature of bacterial cell surfaces is a function of its physiological state. The work presented here explored *quantitatively* the impact of the physiological state of *Pseudomonas aeruginosa* Olin on its surface thermodynamic characteristics and its adhesion to dolomite. Lewis acid/base (hydrophobic), Lifshitz−van der Waals (electrodynamic), and Coulombic (electrostatic) forces were measured via contact angle measurements and electrophoretic mobility assays. It was found that *P. aeruginosa* Olin exhibited a decreased electron-donating potential (γ_i^-) and increased ζ-potential in the stationary phase as compared with logarithmic growth and decay phases. These changes in surface thermodynamic properties were clearly manifested in subsequent partitioning experiments with dolomite. *P. aeruginosa* Olin was found to partition onto dolomite to a significantly larger extent in the stationary phase than in the logarithmic growth or decay phases. This observation further corroborates the need to include Lewis acid/base interactions in the evaluation of bacterium/surface interactions. The reported results indicate the clear impact of physiological state on surface thermodynamics and adhesion.

Introduction

The movement of bacteria through porous geological formations is of paramount importance in the fate and remediation of many polluted surface and subsurface formations. When intrinsic bioremediation is chosen as the remedial action, successful cleanup depends on uniform transport and redistribution of endogenous strains throughout the contaminant plume; when the addition of exogenous strains with specialized metabolic features is favored, their transport from the injection source to and within the contaminant plume is a major factor determining process efficacy. In these cases, prediction of contaminant fate therefore requires accurate incorporation of factors governing bacterial adhesion and transport phenomena. Bacterial transport is also important when the bacteria themselves are of concern; for example, when the organisms are pathogenic or contain a modified genetic element.

Bacterial transport is typically governed by aqueous phase convective movement coupled with retardation through adsorption onto interfaces and straining or trapping in small pores. Adhesion is commonly thought of as the primary retarding factor, while straining is considered important only when the diameter of the bacterium exceeds 5% of the mean pore size (*1*).

When macroscopic bacterial transport through porous media has been studied, the adhesion of bacteria to solid surfaces has typically been modeled phenomenologically with simple linear sorption equilibrium models (*2*, *3*). Factors that impact bacterial adhesion necessarily also impact bacterial transport, such as ionic strength of the aqueous phase (*2*, *4*, *5*), hydrophobicity of bacterial cells (6−8), and surface properties of cells and matrix (*2*, *7*). Adhesion may be mediated by exuded cell products, such as biopolymers as well as fimbriae or spiculations. Critical physicochemical parameters thought to govern adhesion behavior have been estimated or quantified via several different experimental techniques (9−11): electrostatic character has mainly been measured using ζ potentials (*3*, *12*, *13*) and ion exchange chromatography (*7*, *14*); bacterial cell hydrophobicity has been measured using contact angle measurements (*6*, *10*, *13*, *15*), hydrophobic interaction chromatography (*7*, *16*), and partitioning assays (*17*). Moreover, the cell surface changes with physiological state and environmental conditions (*7*, *18*−*21*). Changes in both the hydrophobic and the electrostatic character of the cell surface with altering growth conditions (e.g., transition into stationary phase) have been reported (*7*, *8*, *22*). Several researchers have, as a result, observed that the degree of sorption on a solid surface changes with the culturing conditions prior to or during the experiment (22−26).

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Macroscopic models of microbial transport have, in general, ignored detailed quantitative treatment of interfacial forces between solid surfaces and microbes. Empirically determined collision efficiency factors have been used to accommodate energy barriers that result in less than 100% attachment of particles or microbes to surfaces (*27*). It appears that few models to date have incorporated colloid stability theory such as the classical Derjaguin, Landau, Verwey, & Overbeek (DLVO) theory—which considers van der Waals and electrostatic interactions—and none have incorporated extended DLVO theory—which also considers long-range hydrophobic (Lewis acid/base) forces—into pore scale trajectory models. The work reported here is part of a larger effort to thermodynamically model bacterial adhesion and to estimate prospectively filter coefficients for transport modeling (*28*, *29*). Notwithstanding the obvious dependence of cell surface physicochemistry on physiological status and the impact those factors may have on bacterial transport, such impact has not been systematically explored and quantified in the bacterial transport literature. This paper summarizes the results of the impact of physiological state on the surface characteristics and adhesion of a specific bacterial strain, *Pseudomonas aeruginosa* Olin.

Experimental Protocol

P. aeruginosa strain Olin was obtained from Olin Research Center (Cheshire, CT) and selected to represent an environmentally ubiquitous bacterium. Cells were grown in a minimum mineral salts medium. The mineral salts medium [300 mg/L $(NH_4)_2SO_4$, 14.8 mg/L KNO_3, 840 mg/L $NaHCO_3$, 136 mg/L KH_2PO_4, 30 mg/L $CaCl_2 \cdot 2H_2O$, 34.5 mg/L $MgSO_4 \cdot 7H_2O$, 0.1% succinic acid] was adjusted to pH 7.2 prior to sterilization by autoclaving (121 °C, 1 atm, 20 min). Side-armed flasks (250 mL) containing 50 mL of the medium were inoculated with 1.0% of logarithmic growth cells and incubated in a gyratory water bath shaker (New Brunswick Model G76D) at 32 °C and 150 rpm. At various time intervals, culture density was determined spectrophotometrically (Milton Roy, Spectronic 20D+) via optical density at 660 nm. UV—vis scanning of cell-free supernatant from stationary phase cultures indicated a maximum absorbance at 410 nm in accordance with the wave lengths of maximum absorbance for pyoverdines from fluorescent *Pseudomonas* spp. (*30*, *31*). Subsequent analysis of siderophore production was performed at 410 nm on 2 mL of cell-free supernatant from batch cultures centrifuged at 14000 rpm for 20 min (Eppendorf, Model 5415C).

Physiological states were defined at unique points during bacterial growth in batch culture. Cells grown in mineral salts solution were sampled during mid-logarithmic growth, stationary, and decay phases (OD_{660}:0.239, 0.374, and 0.278) and observed by phase contrast microscopy (Olympus Model BH2) at 1000× magnification.

Cultures were sampled at the three physiological states and subjected to electrophoretic mobility (EPM) assays to measure the Coulombic component of cell surface forces. Laser diffraction techniques using a Malvern Zetasizer II were used to determine EPM in the mineral salts medium. A minimum of six measurements at 120 V under a field strength of 20 V/cm was obtained. EPM measurements were converted to ζ-potentials using the von Smoluchowski equation (*32*).

Surface tension measurements were performed on cultures sampled at the three physiological states to quantify Lewis acid/base and Liftshitz—van der Waals forces. Sufficient aliquots of cell suspensions were vacuum filtered (27 mmHg, 10 min) through porous silver membranes (47 mm i.d., Hytrex) to deposit a uniform lawn of approximately 13 mg of cells (based a developed relationship between OD_{660} and cell DW concentration: g DW/L = 0.326 OD_{660} − 1.98 × 10^{-3}, r^2 = 0.98). Deposited cell lawns were uniformly rinsed with 50—75 mL of deionized water. Upon air-drying for approximately 30 min, a smooth flat layer formed, on which contact angles (θ) were measured (33—35).

Contact angles were measured using water, glycerol, diiodomethane, and formamide as liquids (*L*) on bacterial surfaces (*S*). The use of these liquids allowed the quantification of the various components of surface tension (*35*). A Rame—Hart goniometer was used to acquire a minimum of eight advancing contact angle measurements per sample. Using the van Oss—Chaudhury—Good equation (33—35), various surface tension components were calculated:

$$(1 + \cos\theta)\gamma_L = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{WL}} + \sqrt{\gamma_S^{+}\gamma_L^{-}} + \sqrt{\gamma_S^{-}\gamma_L^{+}}\right) \quad (1)$$

where γ_L is the total surface tension of the liquid (L) (J/m^2); γ_i^{LW} is Lifshitz—van der Waals (LW), or apolar surface tension component of condensed material (i) (J/m^2); γ_i^+ and γ_i^- are the electron-acceptor and -donor parameter (respectively) of the Lewis acid/base (AB), or polar component of the surface tension of condensed material (i) (J/m^2); $\gamma_i^{AB} = 2\sqrt{\gamma_i^+\gamma_i^-}$ and $\gamma_i = \gamma_i^{LW} + \gamma_i^{AB}$ (J/m^2). The term γ_i^{LW} comprises the dispersion as well as the induction and orientation contributions to van der Waals interactions. Details on surface energy calculations are described elsewhere (33—35).

Partitioning experiments with dolomite (Barstow, CA; *36*) and cells sampled at defined physiological states were conducted in 25-mL sample vials closed with screw caps containing Teflon-lined septa placed on a gyratory shaker table at 20 °C. The initial concentration of dolomite was 1.0 mg/mL while initial concentrations of *P. aeruginosa* Olin were 0.076, 0.120, 0.089 mg dry weight (DW)/mL for logarithmic-growth, stationary, and decay phases, respectively. A minimum of five adhesion experiments were conducted for each physiological state. Dolomite was chosen because of its well-documented surface characteristics and to represent a common subsurface material (*36*). Initial adhesion experiments were done in two different manners. In the first technique, cells were harvested at the defined physiological state, centrifuged at 500*g*, resuspended to the same volume in deionized water, and combined with dolomite. In the second technique, aliquots of the batch culture were directly mixed (i.e., in the mineral growth medium) with the dolomite. In the four initial experiments, both approaches were used and compared. Adhesion results were identical for both treatments. All adhesion results reported herein were performed in the mineral growth medium. The bacteria (at various physiological states) and dolomite were equilibrated for 30 min. The duration of the experiment ensured that partition equilibrium was achieved while physiological states were not significantly altered (*37*). Particle size distribution analyses of bacterial and dolomite suspensions were conducted with a HIAC/ROYCO analyzer equipped with a liquid sensor (Model 325e), a syringe-operated sampler

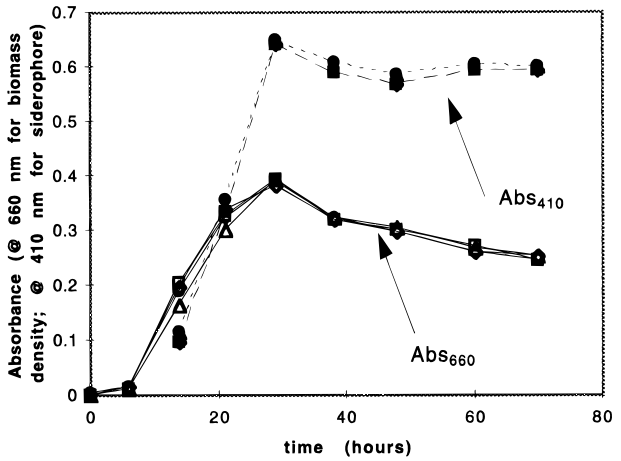

FIGURE 1. Culture density (open symbols, Abs at 660 nm) and siderophore concentration (closed symbols, Abs at 410 nm) for four replicate batch cultures of *P. aeruginosa* Olin in mineral salts medium.

(Model 3000), an optical HIAC/ROYCO particle counter (Model 9064) and a configured computer. Mean diameters of bacterial and dolomite suspensions were measured as 1 and 10 μm, respectively. As a result, at the end of the partitioning assays, the suspension was filtered through 13 mm i.d. 5 μm polycarbonate syringe filters (Millipore) to remove the non-attached bacteria. Each adhesion experiment included two control vials. In one vial, only dolomite was mixed with cell-free medium supernatant; in the other vial, only aliquots of cell cultures were transferred. Initial optical densities of both control vials were measured. Both suspension were subjected to the same treatment as in the adhesion vials and filtered through a 5-μm filter after 30 min. Optical densities of the filtrate for the dolomite-only controls were consistently in the background region, indicating no dolomite bleed-through. Optical densities of the filtrate for cell-only controls were consistently identical ($\pm$ 2%) to the value of the original culture aliquot used, indicating minimal cell density changes during the assay and complete recovery of non-adhered cells. Adhesion in experimental vials was measured by the difference in the OD_{660} of the unfiltered culture aliquots and of the 5-μm filtrate after 30 min.

Results

Culture density and siderophore concentration increased rapidly during the first 30 h of batch growth with the logarithmic phase lasting approximately 10 h (Figure 1). At 30 h, maximum optical densities of 0.394 (at 660 nm) and 0.650 (at 410 nm) were observed for culture density and siderophore concentration, respectively. Siderophore production commenced after approximately 15 h of growth. After approximately 30 h, culture density and siderophore concentration started to decrease, with a faster rate of decrease in the culture density. As a result, the siderophore to biomass ratio increased asymptotically with time. The ratio for the stationary and decay phase cells, however, were very similar. Based on the reported molar extinction coefficient ($\epsilon = 20\,000$ L mol^{-1} cm^{-1}) and molecular weight (1500 $\pm$ 75) for the yellow-green fluorescent siderophore from *Pseudomonas* spp., the maximum siderophore concentrations were estimated to be approximately 5 mg/L (*30*).

EPM determinations are reported in Table 1. Coefficients of variation were no greater than 5%. The mean

TABLE 1

Electrophoretic Mobility Measurements for *P. aeruginosa* Olin in Different Physiological States[a]

physiological state	$EPM_{\bar{x}}$	σ	N
logarithmic	−1.23	0.06	6
stationary	−1.83	0.06	6
decay	−1.28	0.04	6

[a] $EPM_{\bar{x}}$ = mean electrophoretic mobility, μV/cm·s; σ = standard deviation; N = number of measurements.

TABLE 2

Contact Angle[a] Measurements for *P. aeruginosa* Olin in Different Physiological States

	logarithmic			stationary			decay		
liquid	$\theta_{\bar{x}}$	σ	N	$\theta_{\bar{x}}$	σ	N	$\theta_{\bar{x}}$	σ	N
diiodomethane	53.1	1.5	12	56.4	3.2	9	52.6	1.5	10
glycerol	65.0	1.8	12	69.8	4.3	11	56.8	1.8	10
water	33.5	1.2	21	49.8	1.4	11	37.7	1.3	8
formamide							43.2	1.4	14

[a] $\theta_{\bar{x}}$ = mean contact angle; σ = standard deviation; N = number of measurements.

EPM of bacteria in stationary phase was approximately 46% higher than of those in the logarithmic or decay phases. Contact angle measurements are presented in Table 2. The standard deviation of diiodomethane and glycerol contact angles for bacteria in the stationary phase was somewhat higher than for those in the logarithmic or decay phases (Table 2); while the standard deviation for the water contact angle of all examined bacteria was consistently the lowest.

Surface thermodynamic characteristics of *P. aeruginosa* Olin in various physiological states and dolomite are summarized in Table 3. The measured ζ-potential values were at the low end of potentials typically encountered for aquatic particles (*38*). The ζ-potentials for both the logarithmic growth and decay phases were very similar and relatively low. The bacteria exhibited higher values of ζ-potential in the stationary phase than in either the logarithmic growth or decay phases. Approximately 40−50% higher ζ-potentials were observed for stationary phase cells. In this range of ζ-potentials, the Debye−Hückel linear approximation is valid, and the surface potential is directly proportional to surface charge density (*39*).

As expected, the Liftshitz−van der Waals component of surface free energy changed only slightly among the three phases. Values of γ_i^{LW} decreased from 32.54 mJ/m^2 for logarithmic growth to 30.66 mJ/m^2 for bacteria in the stationary phase. For bacteria in the decay phase, γ_i^{LW} increased to 32.83 mJ/m^2. These can be considered relatively insignificant changes.

The Lewis acid/base component of surface tension appeared primarily responsible for stabilization of the bacterial suspension. The electron-donating (γ_i^-) and -accepting (γ_i^+) parameter of the polar surface tension component indicates an essentially monopolar (i.e., $\gamma_i^+ \approx 0.1$ mJ/m^2 and $\gamma_i^- > 28.5$ mJ/m^2 (*40*)) hydrophilic biological surface in all states. The value of γ_i^- decreased 1.5-fold from logarithmic growth to stationary phase. In virtually all polar materials and certainly in those of biological origin, the γ_i^- value is indicative of the material's hydrophobicity (most of these materials have a value close to zero for γ_i^+ when dry) (*41*). Hydrophobicity or hydrophilicity can be

TABLE 3

Various Components of Surface and Interfacial Energies for Dolomite and *P. aeruginosa* Olin in Different Physiological States[a]

surface	ζ-potential (mV)	γ_i^+ (mJ/m^2)	γ_i^- (mJ/m^2)	γ_i^{LW} (mJ/m^2)	γ_{iw}^{LW} (mJ/m^2)	γ_{iw}^{AB} (mJ/m^2)	ΔG_{iwi} (mJ/m^2)
logarithmic	−17.59	0.381	53.70	32.54	1.07	−20.20	38.26
stationary	−26.17	0.211	39.49	30.66	0.75	−11.33	21.16
decay	−18.50	0.035	55.18	32.83	1.12	−23.13	44.02
dolomite[b]	−29.81	0.200	13.60	27.10	0.29	12.54	−25.66

[a] i = solid surface and w = water. [b] Data adapted from van Oss et al. (*36*).

expressed in terms of the free energy of interaction (ΔG_{iwi}) between two molecules (ignoring rather insignificant electrostatic interactions) or particles (i) in water (w) (*33*, *35*, *42*, *43*):

$$\Delta G_{iwi} = -2\gamma_{iw} = -2(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}})^2 - 4(\sqrt{\gamma_i^+} - \sqrt{\gamma_w^+})(\sqrt{\gamma_i^-} - \sqrt{\gamma_w^-}) \quad (2)$$

where γ_{iw} is the interfacial tension of condensed material (i) with water (mJ/m^2)

When $\Delta G_{iwi} > 0$, particle i is hydrophilic and when $\Delta G_{iwi} < 0$, particle i is hydrophobic (*40*). Positive values for ΔG_{iwi} of 38.26, 21.16, and 44.02 mJ/m^2 were obtained for the three physiological states, respectively; cf. Table 3. The degree of bacterial surface hydrophilicity, as expressed by ΔG_{iwi}, decreases significantly in the stationary phase. The transition from logarithmic growth to stationary phase results in decreased hydrophilicity ($\Delta G_{iwi} = 38.26$ mJ/m^2 versus $\Delta G_{iwi} = 21.16$ mJ/m^2), which increases the probability of adhesion of stationary phase bacteria to many relatively hydrophobic surfaces and results in a transition from a free to attached state. In the decay phase, the strains are again more hydrophilic ($\Delta G_{iwi} = 44.02$ mJ/m^2) resulting in a lower probability of adhesion. Observations by phase contrast microscopy revealed that *P. aeruginosa* Olin was planktonic during logarithmic growth while some aggregation occurred with stationary phase cells. This observation corroborates the thermodynamic analysis.

In partitioning assays with dolomite, a five-fold increase in attachment was observed in the stationary phase as compared to the other phases. Mean percent reductions of aqueous phase bacterial concentrations after a 30-min equilibration period with dolomite were 8%, 51%, and 10% for the logarithmic growth, stationary, and decay phases, respectively. Coefficients of variation were no greater than 3%.

Discussion

The observed changes in hydrophobicity measured for *P. aeruginosa* Olin are in contrast to the observed increases in the surface hydrophobicity (as measured by hydrophobic interaction chromatography) of *P. aeruginosa* with increases in the specific growth rate in an Fe(III)-limited medium by Allison *et al.* (*44*). On the other hand, the same authors measured a pattern qualitatively similar to our observations for *Escherichia coli* (i.e., a larger hydrophobicity in the stationary phase than in the logarithmic growth phase) (*45*).

Consistent with the findings of our work, Dufrêne and Rouxhet (*46*) reported an increase in cell hydrophobicity (as measured by water contact angles) of *Azospirillium brasilense* from the logarithmic growth phase to the stationary phase. They also correlated these results with increased adhesion in the stationary phase to two supports (glass and polystyrene). The decay phase was not studied. They reported no change in electrophoretic mobility between the logarithmic growth and stationary phases and concluded qualitatively that traditional DLVO theory could not explain their adhesion results.

Fimbriae and pili, when present, can contribute to the hydrophobic character of the cell, and for some strains the presence of pili appears to be required for adhesion (47−49). It has been reported, for example, that the presence of fimbriae in *S. typhimurium* results in increased hydrophobicity and that fimbriated cells adhere to mineral particles to a greater extent (*7*). In addition, pili of *P.aeruginosa* may contribute to adhesion (*49*). The presence of fimbriae could not be confirmed in the TEM analyses (10 700−26 900× magnification), performed during our work. The reported surface and interfacial energies and electrostatic measurements must therefore be considered average macroscopic properties of the cell surfaces including the impact of fimbriae and other surface heterogenieities, but the relative contribution of fimbriae to the reported results is unclear.

In the growth medium employed during this study, *P. aeruginosa* Olin produced a fluorescent siderophore, most likely pyoverdine *Pa* (*50*). However, it was confirmed (Figure 1) that the production of the compound ceases as cells enter the stationary phase (*51*). The contribution of the siderophore to observed surface and interfacial thermodynamics, if any, is therefore expected to be the same in the stationary and decay phases and cannot explain the significant differences observed between these two phases.

Surface thermodynamic characteristics of dolomite are also summarized in Table 3. Dolomite exhibited a moderate ζ-potential and characteristics of a relatively hydrophobic monopolar base surface. A qualitative inspection of the data presented in Table 1 indicates that electrostatic forces would be more significant in inhibiting the partitioning of *P. aeruginosa* Olin in the stationary phase when compared to the logarithmic growth and decay phases. Consequently, traditional DLVO theory could not explain the adhesion experiment results. On the other hand, the Lewis base component indicates that increased partitioning is expected in the stationary phase when compared to the other phases. The Lifshitz−van der Waals interactions are of equal magnitude among all three phases, imparting no preferential partitioning characteristics. Experimental results indicate that *P. aeruginosa* Olin adheres to dolomite (a hydrophobic surface, $\Delta G_{iwi} = -25.66$ mJ/m^2), to the greatest extent, during the stationary phase.

This observed maximum degree of adhesion during stationary phase is contrary to earlier reports by Fletcher *et al.* (*25*), who reported that adhesion onto polystyrene of a marine pseudomonad was highest in logarithmic phase and decreased in the stationary and decay phases. Similarly,

McEldowney *et al.* (*22*) reported decreased adhesion onto polystyrene with decreasing growth rates for a number of bacterial strains. It has also been reported that cells tend to be more hydrophobic at high growth rates (*3*) which may again lead to higher adhesion to polystyrene.

From a thermodynamic perspective, it appears that cells in the logarithmic growth and decay phases exhibited increased hydrophilic monopolar repulsion, while cells in the stationary phase experienced significantly less hydrophilic repulsion. Hydrophobicity has been reported as being dominant in explaining cell adhesion to surfaces (*6*, *7*, *47*). Clearly, cell surface hydrophobicity is of quantitative significance and is primarily responsible for adhesion in this study. The results of extended DLVO heterocoagulation modeling of dolomite/*P. aeruginosa* Olin interactions will be presented in a forthcoming paper.

Our findings suggest that bacterial transport models may need to be expanded to include the possible changes in cell surface thermodynamics accompanying physiological state changes. Indeed, should the physiological state of bacteria strains of environmental relevance be altered during transport or process implementation, changes in bacterial surface properties may significantly impact adhesion characteristics.

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