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Adhesion of *Pseudomonas putida* onto kaolinite at different growth phases

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ABSTRACT

Bacterial adhesion to minerals in soils and sediments is of fundamental importance in mineral weathering and formation, soil aggregate stability, organic matter degradation and the fate of pollutants. Bacterial surface properties are considered to govern adhesion, and these properties likely change as a function of bacterial growth phase. However, the effect of growth stage on bacterial adhesion to clay minerals remains unclear. This work examined the influence of growth phase on the adhesion of *Pseudomonas putida* to kaolinite-coated coverslips. Fluorescence microscopy, together with a bacterial viability stain, was used to directly quantify surface cell density and viability of adhered *P. putida*. In situ attenuated total reflectance Fourier transform infrared spectroscopy was applied to yield molecular information about the characteristics of the bacteria, and the adsorption and desorption kinetics. Stationary-phase cells exhibited a higher adsorption density on kaolinite surfaces than mid-exponential-phase cells under static deposition conditions. Compared with the mid-exponential-phase cells, the stationary-phase cells displayed higher saturation coverage, and we fitted the results using a pseudo-first-order kinetics equation. The greater extent of adhesion of the stationary-phase cells was probably due to their smaller cell size and less negative surface charges compared with the cells from other growth stages, which resulted in deeper secondary energy minima and lower energy barriers for adhesion. The results from this study suggest that growth phase may strongly influence cell mobility and biofilm formation in aqueous geochemical environments.

1. Introduction

Bacteria are ubiquitous in soils and sediments with densities ranging from 10^7 cells per gram to 10^{10} cells per gram (Kämpfer et al., 1991; Albrechtsen and Winding, 1992; Torsvik and Øvreås, 2002; Yong and Crawford, 2004). Although some of these bacteria are planktonic in solution, most are attached to mineral surfaces (Holm et al., 1992; Nannipieri et al., 2003). Bacterial adhesion is of paramount importance in governing bacterial activities, water–rock interaction, weathering and formation of minerals, soil aggregate stability, organic matter degradation, and fate of pollutants (Kim et al., 2004; Huang et al., 2005; Morrow et al., 2005; Wu et al., 2014a).

Initial bacterial adhesion is considered a main physicochemical interfacial process and may be strongly dependent on cell surface macromolecules, hydrophobicity, charge properties, and solution chemistry. The adhesive nature of bacteria was possibly attributed to features such as lipopolysaccharides (LPS), outer membrane proteins, surface appendages (flagella, fimbriae, or pili), and extracellular polymeric substances (EPS). The molecules of LPS on Gram-negative bacterial cell walls were reported to affect adhesion to disparate solid surfaces including TiO_2, Al_2O_3, SiO_2 (Jucker et al., 1997; Walker et al., 2004), silicon nitride tips of atomic force microscopy (Velegol and Logan, 2002; Abu-Lail and Camesano, 2003; Strauss et al., 2009), silica glass beads (Burks et al., 2003), glass slides, aluminum, stainless steel, polyvinyl chloride, polycarbonate, and polyethylene (Chao and Zhang, 2011). These studies have pointed out that LPS molecules mediated adhesion via steric interactions and hydrogen bonding between O-antigen of LPS and solid surface hydroxyl groups. Growing evidence indicates that cell adhesion is promoted by outer membrane proteins such as the 150 kDa putative iron reductase (Lower et al., 2001, 2005a), protein A (Lower et al., 2005b), cytochromes MtrC and OmcA (Lower et al., 2007), and enterococcal surface proteins (Johanson et al., 2012). The proteins were suggested to function in adhesion through interfacial electron transfer process or the formation of specific bonds with crystallized iron oxides or silicon nitride tips. However, not all outer membrane proteins enhance bacterial adhesion. For example, the adhesion of *Escherichia coli* to quartz sands was inhibited by the outer membrane

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protein TolC at ionic strengths higher than 0.02 mol·L^{-1} NaCl (Feriancikova et al., 2013).

Bacterial EPS includes mainly polysaccharides, proteins, nucleic acids, and lipids (Flemming and Wingender, 2010). Studies show that the presence of EPS on cell surfaces enhances the adsorption of bacteria on glass beads (Liu et al., 2007), silica surfaces (Long et al., 2009), and quartz sands (Tong et al., 2010). The absence of EPS had no apparent effect on the adhesion of *Bacillus subtilis* at low mass ratios of the wet bacteria to minerals (<0.4 for clay minerals and <1.8 for goethite). Interestingly, at high mass ratios, EPS removal decreased adhesion to clay minerals but increased adhesion to goethite (Hong et al., 2013). For EPS-rich bacterial strains, cell adhesion was improved by polymer interaction with glass beads, but for EPS-poor strains, cell adhesion was suppressed by electrostatic interactions with the beads (Tsuneda et al., 2003). The roles of individual compositions of EPS in bacterial attachment are different. Phosphodiester groups of extracellular DNA were found to form innersphere coordination with surface Fe atoms on crystallized iron oxides during adhesion of bacteria and EPS (Omoike and Chorover, 2006; Parikh and Chorover, 2006). Colanic acid was shown to inhibit *E. coli* cell adhesion to inert substrates (Hanna et al., 2003; Chao and Zhang, 2011). Removal of bacterial surface polysaccharides led to decreases in the average adhesion force of *Pseudomonas putida* with silicon nitride tips by about half (Bell et al., 2005).

Besides surface polymers, cell hydrophobicity (van Loosdrecht et al., 1987a), charge properties (van Loosdrecht et al., 1987b), and solution chemistry (Hong et al., 2014; Wu et al., 2014b) are thought to affect bacterial adhesion. Cell surface appendages, such as flagella and fimbriae or pili, have been observed to improve bacterial attachment to hydrous ferric oxide (Caccavo and Das, 2002); quartz sands (Haznedaroglu et al., 2010); six inert surfaces (Chao and Zhang, 2011); fluorinated, amine-terminated, 2-(methoxypolyethyleneoxypropyl)trimethoxysilane (PEG)-like monolayers; silicon wafer; and mica (Xu et al., 2013). The roles of cell appendages in bacterial adhesion may be attributed to their contributions to cell mobility and hydrophobicity. The adhesion amount of *Salmonela typhimurium* to quartz, albite, feldspar, and magnetite was correlated positively with the hydrophobicity and positive charge of the cells (Stenström, 1989). The amount of *P. putida* adsorbed to kaolinite, montmorillonite, and goethite decreased with increasing pH (3–10) and organic acid ligands (0–0.08 mol·L^{-1}), and decreasing salt concentrations (0.05–0 mol·L^{-1}; Jiang et al., 2007; Rong et al., 2008, 2010; Wu et al., 2011). The increase of pH (4–9) or decrease of salt concentrations (0.1–0 mol·L^{-1}) inhibited *P. putida* attachment to soil particles of different sizes (Wu et al., 2012). These results gave critical insight into the importance of electrostatic interaction and ligand-competitive binding in bacterial adhesion to soil active particles.

Bacteria are inherently dynamic organisms, and their protein coverage, EPS coverage and LPS molecule conformation, hydrophobicity, and charge properties evolve as a function of growth phase and are likely contribute to the chemical heterogeneity of the bacterial surface. The stationary growth cells of *E. coli* had a more heterogeneous distribution of charged functional groups on the bacterial surface than the mid-exponential cells, which resulted in higher attachment to quartz surfaces (Walker et al., 2005a,2005b). Naturally, surfaces exposed to bacteria in soils and sediments include primary minerals, clay minerals, and organic matter. Clay minerals are the most reactive inorganic colloid components in soils and sediments. The interactions of bacteria with clay minerals govern a variety of physical, chemical, and biological processes including aggregation, colloid properties, nutrient cycling, microbial ecology, and behavior of contaminants in soil and associated environments. Quantifying the extent of bacterial adhesion to clay minerals has been particularly challenging because of the difficulties of physical separation of bacteria from clay minerals, both of which are micron-scale objects of similar size. Therefore, limited information is available regarding the effect of growth phase on bacterial adhesion to clay minerals, although plenty of data have been reported for bacterial adhesion to primary minerals.

The objective of this paper is to examine the role of growth phase in the adhesion of *P. putida* to kaolinite-coated coverslips. Kaolinite is used as a representative 1:1 layer-type clay mineral, which is the major clay mineral in tropical and subtropical soils. *P. putida* is a Gram-negative aerobic species naturally occurring in soils. LIVE/DEAD staining in combination with fluorescence microscopy allows us to examine the surface cell density and viability of adhered bacteria. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was performed to probe the adsorption and desorption kinetics and the molecular characteristics of the attached cells. Cell characterization techniques were further employed to delineate the extent to which growth phase alters cell surface properties and how this ultimately affects bacterial adhesion. Understanding how growth phase mediates cell adhesion is of vital importance in major biogeochemical processes including biofilm formation, microbial transport, mobility, and ultimate fate of contaminants bound to microbial surfaces.

2. Materials and methods

2.1. Preparation of kaolinite-coated coverslips

The mineral obtained from The Clay Minerals Society was a well-crystallized kaolinite (KGa-1b) from Washington County, Georgia. To attach a layer of clay to glass coverslips (22 mm × 22 mm, 0.13 mm to 0.16 mm thick; Ted Pella, Inc.), the procedure developed by Bickmore et al. (1999) was modified. Poly(ethyleneimine) (PEI; MW, 1300; Sigma-Aldrich) was diluted 1:2000 by mass with deionized water (18.24 MΩ·cm). The coverslips were immersed in the PEI solution for 30 s, rinsed continuously with deionized water for 5 min, oven-dried at 90 °C for 1 h, and allowed to cool. Clay mineral suspensions (1 g·L^{-1}) were prepared using deionized water, and the pH value of the suspensions was regulated to 10.0 with the addition of NaOH and HCl. The suspensions were disaggregated using a sonic dismembrator (Branson Sonifier 450) for 1 min at ~160 W. To prepare the <2 μm colloid fraction, the ultrasound-pretreated suspension was agitated by shaking for ~30 s and left to settle for 3 h and 22 min at 26 °C. This step allows for settling of the >2 μm particle fraction, with the <2 μm particle fraction found in the top 5 cm of the suspension. Using a pipette, the colloid fraction was removed from the 0 cm to 5 cm layer. After heating the coverslips to ~120 °C, 1.0 mL of the colloid suspension (0.475 mg·mL^{-1}) was pipetted onto the coverslip and left for 20 min to boil the clay onto the glass substrate. The coverslips were rinsed continuously with ultrapure water for 20 s and dried in a vertical flow clean bench.

2.2. Bacterial growth and preparation

P. putida X4 (CCTCCM209319) was preserved at the China Center for Type Culture Collection (CCTCC; http://www.cctcc.org/). The cells were grown in Luria-Bertani broth at 28 °C and 180 rpm until the desired growth phase was reached (14 and 40 h corresponding to mid-exponential and stationary phases, respectively), at which time they were harvested for use. The cells were pelleted by centrifugation for 10 min at 4075 *g* and 10 °C. The growth medium was decanted, and the pellet was rinsed three times with 0.01 mol·L^{-1} NaCl at a pH of 5.6 to 5.9. The resulting pellets were resuspended in 0.01 mol·L^{-1} NaCl. The cell density of bacterial suspensions was determined by optical density at 600 nm ($OD_{600\ nm}$). The colony-forming unit per milliliter (CFU·mL^{-1}) at $OD_{600\ nm}$ was measured by a dilution-spread-plate method. To obtain a series of cell densities, the prepared cells were diluted with the electrolyte solution, and actual densities after dilution were verified by spectrophotometry. Viability tests for the mid-exponential and stationary phase cells were performed using a commercial kit (L7012, LIVE/DEAD BacLight Bacterial Viability Kit,