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The effect of extracellular polymeric substances on the adhesion of bacteria to clay minerals and goethite

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ABSTRACT

The functions of extracellular polymeric substances (EPS) during the adhesion of *Bacillus subtilis* to kaolinite, montmorillonite, and goethite were examined by a direct comparison of the adhesion behaviors of native and EPS-free cells *via* cation exchange resin (CER) treatment using batch experiments, attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy, and potentiometric titration. The EPS removal had no apparent influence on bacterial adhesion when the wet bacteria/mineral mass ratio was low (<0.4 for the clay mineral systems and <1.8 for the goethite system). With higher mass ratios, the absence of EPS reduced adhesion to clay minerals but enhanced adhesion to goethite. The ATR-FTIR spectra suggested that protein conformational changes were involved in the adhesion of bacteria to clay minerals, whereas additional chemical interactions such as P–OFe bonds were important for adhesion to goethite. In addition to electrostatic forces (repulsion for clays and attraction for goethite), absence of chemical interactions may also cause the relatively much weaker bacterial adhesion to clay minerals than to goethite. The absence of EPS did not change the interaction mode of the adhesion to clay minerals but enhanced the chemical interactions *via* carboxyl groups for bacteria–goethite adhesion. The potentiometric titration results coupled with the ATR-FTIR spectra showed a significant increase in site concentrations of the CER-treated bacteria as compared to the native cells. Changes in surface site concentrations and chemical interactions that were accompanied by the EPS removal may reasonably explain the influences of EPS on bacterial adhesion to different minerals.

1. Introduction

The adhesion of microorganisms to solid surfaces is ubiquitous in nature; as much as 97% of approximately 10^{30} prokaryotes on Earth are associated with minerals in aqueous solutions, rather than being present as plankton cells (Nannipieri et al., 2003; Kendall and Lower, 2004). This interaction has great significance in biofilm formation (van Loosdrecht et al., 1990; Beveridge et al., 1997; Donlan, 2002), microbial activity, and microbial survival (Marshall, 1975; Beveridge et al., 1997), as well as the transport of microorganisms (*e.g.*, pathogens) (Mills et al., 1994; Chen et al., 2008) and contaminants such as heavy metals (Yee and Fein, 2002) in soil and aquatic environments.

The adhesion of bacteria to solid substrates can be considered an abiotic physicochemical process that is consequently governed by bacterial surface properties such as their specific surface area, electrical properties, hydrophobicity and surface functionalities (Bos et al., 1999; Hermansson, 1999). These surface properties and the resulting adhesions have been extensively reported to be strongly dependent on the solution chemistry (*e.g.*, pH and ionic strength) (Mills et al., 1994; Hermansson, 1999; Jiang et al., 2007) and the sorbent material (Mills et al., 1994; Ams et al., 2004) (*e.g.*, iron oxides).

As the major component of bacterial exterior surfaces, extracellular polymeric substances (EPS), which are a heterogeneous mixture of polysaccharides, proteins, nucleic acid, and lipids, may likewise participate in adhesion processes. Covalent bond formations during the adsorption of EPS isolated from bacteria and Fe-oxy(hydr)oxides have been demonstrated by spectroscopic investigations using Fourier-transform infrared (FTIR) and X-ray absorption fine structure spectroscopy (Omoike and Chorover, 2004, 2006; Fang et al., 2012). Atomic force microscopy (AFM) and electronic structure calculations indicated the formation of hydrogen bonds between the phosphate groups of EPS and the neutral silanols on silica (Kwon et al., 2006). Several studies provided preliminary evidences of the effects of EPS on bacterial adhesion to minerals. Quartz crystal microbalance with dissipation revealed a decline in the deposition efficiencies for *Escherichia coli* BL21, *Pseudomonas* sp. QG6, *Rhodococcus* sp. QL2, and *B. subtilis* on silica surfaces after EPS removal *via* cation exchange resin (CER) treatment regardless of cell type and motility (Long et al., 2009). The direct force measurements using AFM showed that

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adhesion forces between the three species of leaching microorganisms, namely, *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*, and the mineral chalcopyrite decreased after the bacterial EPS was removed using ethylenediaminetetraacetic acid (EDTA) (Zhu et al., 2012). However, these studies focused on adhesion only to primary minerals such as silica. The functions of EPS in cell adhesion to clay minerals and oxides, which are the most common solid surfaces exposed to bacteria in soils, have not been unambiguously demonstrated. To our knowledge, the role of EPS in cell adhesion to phyllosilicate has never been explored.

The current study aims to investigate the contribution and mechanism of EPS during bacterial adhesion to several representative soil minerals. The adhesions of both native and CER-treated *B. subtilis* to kaolinite, montmorillonite, and goethite were investigated using batch experiments coupled with *in situ* attenuated total reflectance (ATR)-FTIR spectroscopy and potentiometric titration methods. Different adhesion mechanisms for clay minerals and goethite, and opposite effects of EPS on the adhesions were found and discussed.

2. Materials and methods

2.1. Minerals and bacteria

Two clay minerals (kaolinite and montmorillonite) and one oxide (goethite) were used in the current study. Kaolinite (Shanghai Wusi Chemical Reagent Company, Shanghai, China) and montmorillonite (Henan Xinyang Montmorillonite Company, Xinyang, China) were prepared following previously outlined procedures (Hong et al., 2012). The clays were oxidized using H_2O_2 to remove any residual organic matter. The colloidal fraction (<2μm) of the minerals was collected through sedimentation for use in all experiments. Goethite was synthesized according to Atkinson et al. (1967), and identified using X-ray diffraction. All the prepared minerals were ground to pass through a 100-mesh sieve and stored for subsequent use.

The Gram-positive, EPS-producing *B. subtilis* (Omoike and Chorover, 2006; Cao et al., 2011) that is naturally found in soil was used in this study. Bacteria were cultured, harvested, and cleaned according to the protocols described in the Supporting information. The cells were resuspended in ultrapure water and then divided into two portions. One portion of the cell suspension was stored as untreated bacteria with EPS. The other portion of the cell suspension was used to prepare EPS-free bacteria *via* CER treatment (Frølund et al., 1996), which is presented in detail in the Supporting information. Previous studies (Tourney et al., 2008; Fang et al., 2011) and experiment using scanning electron microscopy (Fig. S1) have shown that this method can effectively remove the EPS from cells without causing any significant damage to bacterial surface, and that the wash treatment did not significantly alter the cell wall structure and most of the cells were intact. The cleaned cells were isolated by centrifugation and their wet weight was measured. The wet weight of the bacterial pellet was approximately ten times the dry weight.

Particle size and selected surface properties such as specific surface area and zeta potential (at pH7 and 1mM ionic strength) of the bacteria and the minerals were determined in our previous study (Hong et al., 2012).

2.2. Bacterial adhesion to minerals

Batch experiments were conducted to measure the isothermal adhesion of both untreated and CER-treated *B. subtilis* to the minerals as a function of the bacteria/mineral mass ratio at pH7.0 and 25°C in a 1mM KNO_3 solution. Procedures for cell adhesion and the separation of free cells from those attached to minerals have been elaborated in previous studies (Hong et al., 2012) and in the Supporting information. In brief, the mixture of bacterial and mineral suspensions was shaken at 200rpm and 25°C in a rotary shaker for 2h. After the adhesion equilibrium, the free cells were separated from the attached cells and mineral particles by injecting a certain volume of sucrose solution (60% by weight) into the bottom of the mixture in the centrifuge tube and centrifuging (Yee et al., 2000). The concentrations of unattached bacteria above the sucrose layer were determined spectrophotometrically at 420nm. The adhesion was calculated by substracting the final unattached bacterial weight from the initial wet weight.

2.3. ATR-FTIR spectroscopy

ATR-FTIR spectroscopy permits *in situ* investigations of functional groups that are involved in bacterial adhesions to minerals in the presence of water (Parikh and Chorover, 2006; Ojeda et al., 2008b). The ATR-FTIR spectra for the bacteria, minerals, and their complexes were collected over the range of 4000cm^{-1} to 600cm^{-1} using a ZnSe internal reflection element (IRE) (Vertex 70, Bruker Optics, Germany). Each sample was scanned 256 times with 4cm^{-1} resolution. The bacteria–mineral complexes were prepared after the adhesion experiment (the bacteria/mineral mass ratio was 0.8, 0.6, and 1.2 for kaolinite, montmorillonite, and goethite, respectively, according to their different adhesions with bacteria). Bacteria and the respective minerals were suspended in a 1mM KNO_3 solution at pH7. After centrifugation at 8000×*g* for 15min, all the wet samples were spread on the IRE crystal to obtain their spectra. The supernatants were scanned as the background spectra.

2.4. Potentiometric titration of bacteria and data analysis

Potentiometric titrations of concentrated (20±1mgwetweightml^{-1}, wet/dry mass ratio was approximately 10) bacteria in 1mM KNO_3 were done according to the procedures described by Fein et al. (2005). Acid–base titrations were performed under a N_2 atmosphere at 25°C using an automatic potentiometric titrator (Metrohm titrator 836, Switzerland). The bacterial suspensions were first titrated to pH2.5 using HCl solutions and then to pH10.0 using NaOH solutions. The concentrations of these HCl and NaOH solutions were approximately 0.1M each, which were standardized against reagent-grade Na_2CO_3 and KH phthalate, respectively. At each titration step, a stability of 0.01mVS^{-1} was attained before adding the next drop of the titrant. The blanks that contained 1mM KNO_3 were titrated under the same conditions. Each titration was performed at least in duplicate.

To determine the acidity constants and concentrations of proton-active functional groups on the bacterial surface, the potentiometric data were fitted to a non-electrostatic surface complexation model (Fein et al., 2005). The bacterial surface functional groups were acidic and discrete. The deprotonation of a given group$>R_iH$ can generally be expressed as:

$$>R_iH = >R_i^- + H^+ \quad (1)$$

where $>R_i$ is the *i*th site on the bacterial surface. The acidity constant k_i for reaction (1) can be given by:

$$k_i = \frac{[>R_i^-][H^+]}{[>R_iH]} \quad (2)$$

where $[>R_i^-]$ and $[>R_iH]$ represent the concentration of deprotonated and protonated sites, respectively, while $[H^+]$ represents the proton concentration in the bulk solution.

Based on the mass balance and charge conservation, the final equation for the model was obtained:

$$C_a - C_b + [OH^-] - \left[H^+\right] = -T_H^0 + \sum_{i=1}^{n} \frac{[>R_i]_{tot}}{1 + [H^+]/k_i} \quad (3)$$