# Effects of humic acid on adhesion of *Bacillus subtilis* to phyllosilicates and goethite

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## ARTICLE INFO

## ABSTRACT

Bacterial attachment is a physicochemical interfacial process probably affected by the presence of humic acid (HA) in natural environments. However, the effect of HA coating on bacterial adhesion to soil minerals remains unknown, despite many studies focusing on cell attachment to pure or iron oxide-coated quartz in the presence of HA. The influence of HA on *Bacillus subtilis* adhesion to kaolinite, montmorillonite, and goethite under neutral pH and 1 mM ionic strength was examined using batch experiments coupled with Fourier-transform infrared spectroscopy (FTIR), isothermal titration calorimetry, and Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory. Results showed a reduction in cell adhesion on goethite and montmorillonite with increasing HA content from 0% to 2%, indicating an inhibitory effect of HA coating on the interaction. However, a promotive effect of HA was observed for kaolinite, which disagreed with previous findings based on cell–quartz systems. In addition, the presence of HA reduced the exothermic adhesion enthalpy for kaolinite and goethite, but increased that for montmorillonite. For goethite, the HA coating depressed its positive charge and increased its aggregation, thereby leading to a weaker electrostatic attraction and lesser available surface area, which probably contributed to the observed decreasing adhesion. For montmorillonite, the reduced attachment was likely caused by the increase in electrostatic repulsion that overcompensated for the appeared chemical interactions as suggested by FTIR analysis. The enhanced cell adhesion on kaolinite, which was unexpected from DLVO theory based on its surface charge change, is likely ascribed to the HA-induced decrease in its aggregation. Additionally, the effect of HA vanishes at HA contents higher than 2%, likely due to a saturation effect. Overall, the influence of HA on bacterial attachment is likely a combined result of multiple factors, including not only electrostatic forces and chemical interactions, but also mineral aggregation.

## 1. Introduction

Most microorganisms in soils and sediments are associated with minerals, rather than being planktonic in aqueous solutions (Nannipieri et al., 2003; Kendall and Lower, 2004). The microbe–mineral association plays an important role in a wide variety of biogeochemical and environmental processes, such as biofilm formation, mineral formation and weathering, organic contaminant biodegradation, and pathogen and heavy metal transport (Beveridge et al., 1997; Chenu and Stotzky, 2002; Yee and Fein, 2002; Chen et al., 2008).

The initial adhesion of bacteria to minerals has been investigated over the past decades since the 1970s (Marshall et al., 1971). It is mainly a physicochemical interfacial process determined by the surface physical and chemical properties of both bacteria and minerals, such as electrical properties, specific surface area, hydrophobicity, surface roughness, and surface functional groups (Bos et al., 1999; Hermansson, 1999; Parikh and Chorover, 2006; Hong et al., 2012; Wu et al., 2014). Bacterial adhesion is essentially governed by the forces between them, including Lifshitz–van der Waals interactions, electrostatic force, acid–base interactions, Lewis acid–base interactions, and steric interactions (van Oss, 1995; Bos et al., 1999; Hermansson, 1999). Aside from these physical interactions, chemical bonds have been shown to mediate the associations of bacteria with iron oxides in recent literature (Parikh and Chorover, 2006; Elzinga et al., 2012; Hong et al., 2013; Parikh et al., 2014). Hence, changing the surface properties and the interactions may lead to a variation in bacterial adhesion. For example, removing extracellular polymeric substances (EPS) on the cellular surface alters the bacterial surface charge and chemical interactions, inhibiting the adhesion of *Bacillus subtilis* to kaolinite and montmorillonite, but enhancing its adhesion to goethite (Hong et al., 2013). Similarly, coating bacteria with iron oxides results in a large reduction in the negative charge of quartz and strongly increases the deposition of bacteria (Mills et al., 1994; Knapp et al., 1998; Kim et al., 2008).

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In natural environments, minerals rarely exist with bare surfaces and are smeared with other reactive substances, including humic acid (HA). As a major constituent of natural organic matter, HA is ubiquitously present in soils, sediments, and aqueous environments, and it has been reported to interact with minerals, especially iron oxides, through surface complexation (Balcke et al., 2002; Weng et al., 2006; Yue et al., 2009). The strong interactions of HA modify the surface characteristics of minerals, thereby affecting their attachments with bacteria. The importance of HA on bacterial adhesion has received considerable research attention in the past decades. A study using three representative bacteria *Rhodococcus* sp. QL2, *Escherichia coli* BL21, and *E. coli* C3000 showed that the presence of HA in solution inhibits cell attachments on quartz sand regardless of cell type (Gram-negative or Gram-positive), mobility, the presence or absence of EPS on cell surface, and solution chemistry (ionic strength and ion valence) (Yang et al., 2012). The negative role of HA in bacterial adhesion was also observed in experiments using other bacterial strains and quartz coated with iron oxides (Johnson et al., 1996; Foppen et al., 2008; Park and Kim, 2009). For example, HA reduces the attachment of *E. coli* ATCC 25922 to goethite-coated quartz because HA alters the mineral surface charge and causes steric hindrance to cells (Foppen et al., 2008). Our recent work showed that both Ultisol and Alfisol soil colloids oxidized by $H_2O_2$ adsorbed a lesser amount of bacteria than those with natural organic matter, suggesting a suppressive role of soil organic matter in bacteria attachment with soil particles (Wu et al., 2012; Zhao et al., 2014).

Although most previous studies indicated a significant inhibitory effect of HA on bacterial adhesion, a few studies (Parent and Velegol, 2004) found a negligible role of HA in *E. coli* adhesion to silica or glass surfaces. The influence of HA on bacterial attachment and its underlying mechanism remains unclear. Moreover, most of these studies focused on cell adhesion to pure or iron oxide-coated quartz, rather than to clay minerals and oxides, which are the most common inorganic surfaces exposed to bacteria in natural environment. These minerals have a more reactive and complex surface than primary minerals, and exhibit much greater adhesion than the latter (Hong et al., 2012). Thus, HA may affect bacterial adhesion to clay minerals and oxides differently from bacterial adhesion to primary minerals. However, limited information is available about the effect of HA on cell attachment to clay minerals and oxides.

The current study aimed to determine the influence of HA coating on bacterial adhesion to several representative soil minerals. The adhesion of *B. subtilis* to pure and HA-coated kaolinite (1:1 layer-type clay mineral), montmorillonite (2:1 layer-type clay mineral), and goethite were comparatively investigated using batch experiments coupled with Fourier-transform infrared spectroscopy (FTIR), isothermal titration calorimetry, and Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory. Different effects of HA on bacterial adhesion were found for different minerals, and the possible mechanisms for the discrepancies were discussed.

## 2. Materials and methods

### 2.1. Minerals, bacteria, and HA solution

Two phyllosilicates (kaolinite and montmorillonite) and one oxide (goethite) were used in this study. The colloidal fraction (<2 μm) of kaolinite (Shanghai Wusi Chemical Reagent Co., Ltd., Shanghai, China) and montmorillonite (Henan Xinyang Montmorillonite Company, Xinyang, China) were prepared according to previously outlined procedures (Hong et al., 2012) and used in subsequent experiments. Goethite was synthesized according to Atkinson et al. (1967). All the prepared minerals were identified using X-ray powder diffraction (Fig. S1), ground to pass through a 100-mesh sieve, and stored until use.

*B. subtilis*, a Gram-positive, aerobic strain was employed in this study. Bacteria were cultured, harvested, and cleaned according to the protocols described in our previous study (Hong et al., 2012). Cells were quantified in wet weight, which was approximately ten times the dry weight, and

1 mg mL$^{-1}$ fresh biomass approximated $10^8$ cells mL$^{-1}$. The fresh weight conversions to the cell dry weight and density are described in the Supporting Information.

Dissolved HA was used for mineral surface coating in the present study. HA powder (Shanghai Jufeng Chemical Reagent Co., Ltd., Shanghai, China) was dispersed in ultrapure water (18.24 M Ω cm) to obtain an initial concentration of 1 mg mL$^{-1}$. The HA suspension was adjusted to pH 7 using small aliquots of NaOH solution, at which the HA solubility was higher than 50% (Fig. S2) to ensure sufficient dissolved HA for subsequent use. After shaking at 180 rpm for 24 h, the neutral pH suspension was transferred to a weighed tube and centrifuged at 8000 ×*g* for 10 min. The supernatant was transferred to a clean vessel, and the residual HA was oven dried (105 °C, 24 h) and weighed. The HA concentration in solution was calculated as the weight difference between the initial added HA and the final undissolved fraction. The elemental composition and selected properties of HA are given in Table S1.

### 2.2. Preparation of HA–mineral complexes and measurement of their zeta potential and hydrodynamic diameter

HA–mineral complexes were prepared in a manner similar to HA–mineral adsorption experiments (Li et al., 2003). A certain amount of HA solution was mixed with 1 g of minerals to yield an HA/mineral mass percentage from 0.5% to 4% according to the content of soil organic matter in natural soils. The mixtures were shaken at 180 rpm for one week to reach equilibrium (Li et al., 2003). The HA–mineral complexes were centrifugally separated from the supernatant, cleaned by several washings using ultrapure water, and stored for subsequent use. The HA concentration in the supernatant (including loss during the washings) was measured spectrophotometrically at 450 nm, and the amount of HA bound to mineral was then calculated.

The electrophoretic mobility and hydrodynamic diameter of the HA–mineral complexes (0%–4% HA content) in 10 mM KNO$_3$ solutions (pH 7) were measured using microelectrophoresis and dynamic light scattering with a zeta potential analyzer (ZetaPlus, Brookhaven Instruments, Holtsville, NY, USA), respectively. Zeta potentials were calculated from electrophoretic mobility according to the Smoluchowski equation. The average diameters were evaluated based on the size–number distribution.

### 2.3. Bacterial adhesion to HA-modified minerals

Batch experiments were conducted to measure the isothermal adhesion of *B. subtilis* on both pure minerals and HA–mineral complexes bearing different percentages of HA at pH 7.0 and 25 °C in 1 mM KNO$_3$ solution. Protocols for the measurement of cell adhesion and the separation of free cells from the attached ones have been elaborated in our previous studies (Hong et al., 2012). In brief, varying amounts of bacteria (0–120 mg for HA–phyllosilicate systems and 0–300 mg for HA–goethite systems) were mixed with 100 mg of HA–mineral complex in suspension. The mixtures were shaken at 200 rpm and 25 °C in a rotary shaker for 2 h (Jiang et al., 2007). The pH of the experimental system was measured and adjusted to pH 7, but only minor adjustments were necessary during the experiment. After reaching 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. The concentrations of unattached bacteria above the sucrose layer were determined spectrophotometrically at 420 nm. Adhesion was calculated by subtracting the final unattached bacteria weight from the initial wet weight.

### 2.4. FTIR spectroscopy

The FTIR spectra for the single HA, *B. subtilis*, minerals, and their binary (HA–mineral) and ternary (bacteria–HA–mineral) complexes