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Interaction of photosynthetic bacterium, *Rhodopseudomonas Palustris*, with montmorillonite clay

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Abstract

In this study, we investigate the interaction of *Rhodopseudomonas Palustris* (*R. Palustris*) with montmorillonite clay. The adsorption of bacteria on the clay surface was also determined as a function of the initial bacterial quantity, pH, temperature and ionic strength. At different initial bacterial quantities, the percentage of bacteria adsorbed ranged from 61.07% to 77.57%, and the higher percentage was determined to be 15.0×108 cfu ml^{-1}. In addition, the actual number of adsorbed cells was significantly correlated with the initial quantity of *R. Palustris*. A greater degree of *R. Palustris* adsorption on the montmorillonite was observed in the temperature range of 30oC to 40oC. It was also found that as the pH and ionic strength increased the percentage of bacterial adsorption on montmorillonite decreased. There were no significant differences ($P > 0.05$) in the enzyme activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) between the bacteria treated with and without montmorillonite. However, montmorillonite supplementation significantly increased ($P < 0.05$) the cell surface hydrophobicity of *R. Palustris* compared with the control. These results indicated that the interaction was controlled by physicochemical characteristics between cells and the mineral substrate.

Keywords: montmorillonite; *R. Palustris*; interaction; bacterial adsorption; clay

1. Introduction

Microorganisms comprise much of the Earth's biodiversity and have a critical role in biogeochemistry cycles as well as ecosystem functioning (Hattori, 1973). Microbes, especially bacteria, influence the kinetics and course of reactions involving the dissolution of a number of minerals, and they also influence the authigenic and diagenetic formation of a number of minerals in the lithosphere and hydrosphere of Earth (Ehrlich, 1996). On the other hand, minerals profoundly influence the survival, activity, gene expression and functions of microbes (Xie and Znang, 2001; Manini and Luna, 2003; Chaerun *et al*., 2005). Therefore, the interactions between minerals and microorganisms are essential to soil ecology and to the environment (Rong *et al*., 2007).

Experimental studies have demonstrated that pH, temperature, ionic strength, bacterial species and mineralogy strongly influence the extent of bacterial adsorption onto mineral surfaces (Loosdrecht *et al*., 1989; Scholl *et al*., 1990; Mills *et al*., 1994; Yee *et al*., 2000; Jiang *et al*., 2007; Rong *et al*., 2008). The adsorption of the bacterium, *Bacillus subtilis*, onto corundum increases with decreasing pH, increases with bacterial quantity, increases the mineral mass ratio, and decreases the ionic strength (Mills *et al*., 1994). The adsorption of *Pseudomonas putida* on minerals, including montmorillonite, kaolinite and goethite, decreased with increasing pH, and greater adsorption was observed in the temperature range of 15°C to 35°C (Jiang *et al*., 2007). Gram-positive *B. subtilis* adsorbs onto Fe-oxyhydroxide-coated quartz to a greater extent than do gram-negative *Pseudomonas mendocina* (Ams *et al*., 2004). These studies point to the importance of sorption, or the nonspecific association between the microorganisms and mineral surfaces.

Purple non-sulphur photosynthetic bacteria, such as *Rhodopseudomonas palustris* (*R. Palustris*), are widely distributed in nature (Oda *et al*., 2003; Zhou *et al*., 2007). These bacteria have the potential to be very useful and play a major role in purifying the environment because they combine photosynthesis with the ability to photometabolize many organic substances (Kobayashi, 1982; Getha *et al*., 1998). Higher incorporation rates (80%) of total glucose assimilation in a salt marsh have been observed in bacteria attached to suspended clay minerals to a greater extant than in unattached bacteria (Hanson and Wiebe, 1977; Hanson and Synder,

1980). This observation indicated that bacteria attached to clay have a greater impact than unattached bacteria on the trophic dynamics of some aquatic systems (Wang *et al*., 2005). However, few studies have focused on the adsorption of *R. Palustris* on clay minerals, which are the most active inorganic colloidal components in soils, and the associated interaction mechanisms remain unknown. In the present study, the interaction of the gram-negative bacterium, *R. Palustris*, with montmorillonite clay was investigated. The effects of temperature, pH and electrolytes on *R. Palustris* adsorption were also examined.

2. Materials and Methods

2.1 Bacterium

The bacterium, *R. Palustris*, was previously isolated in our laboratory from shrimp (*Penaeus vannamei*; Boone, 1931) pond sludge in Haiyan, Zhejiang province, China (Wang *et al*., 2004). The Hungate roll tube technique for anaerobic culture of bacterium (Hungate, 1968) was applied in the preparation of media, originally described by van Niel (1971), for *R. Palustris*. The tubes were completely filled with the malate-basal medium (4.0g malic acid, 1.0g NH_4Cl, 0.2g $MgSO_4 \cdot 7H_2O$, 0.2g K_2HPO_4, 0.05g $CaCl_2 \cdot 2H_2O$, 0.05g $FeCl_3 \cdot 6H_2O$, 0.01g $ZnSO_4 \cdot 7H_2O$, 0.01g H_3BO_4, 0.5mg $CuSO_4 \cdot 5H_2O$, 0.5 mg $MnCl_2 \cdot 4H_2O$, 5.0mg $Co(NO_3)_2 \cdot 6H_2O$, 0.1mg *p*-aminobenzoic acid, 0.001mg thiamine-HCl, 0.1mg nicotinic acid and 0.001 mg per liter biotin; pH 6.8-7.0), closed tightly and incubated at 30 ^oC. The specimen of *R. Palustris* was maintained in malate-basal medium with 20% sterile glycerol and stored at -80 ^oC (Forma 702, Thermo, USA) for later use.

Prior to the adsorption assay, *R. Palustris* was serially transferred twice in the malate-basal medium and incubated anaerobically at 30^oC for 72 h. Cells were collected by centrifugation (4000 *g*, 10 min) and washed three times in sterilized distilled-deionized (DDI) water according to Jiang *et al*. (2007). Previous work in this laboratory indicated that the wash procedure does not significantly alter the cell wall structure (Yee and Fein, 2002). The quantity of the washed bacterial cell suspension was determined using the double-plate method (Sun *et al*., 2001). All experiments were conducted in nutrient deficient conditions where bacteria are metabolically inactive.

2.2 Mineral

The montmorillonite used in this study was a hydrothermal product of volcanic sedimentary rocks from Chifeng, in the Inner Mongolia Autonomous Region of China. Besides montmorillonite, there were minor quantities of quartz and volcanic glass in the ore. To remove impurities, the raw material was dried in an oven overnight at 80^oC and milled to less than 300 mesh. The milled material was dispersed in water to form a 10% suspension that was churned in a stirrer for about 10 min. Particles larger than 2 μm were separated out by sedimentation, and the suspension was centrifuged to obtain refined montmorillonite (Xia *et al*., 2004). The refined montmorillonite was dried at 80 ^oC followed by another milling to less than 300 mesh for use. The formula of the purified montmorillonite was as follows $[Na_{0.158}K_{0.082}Ca_{0.256}Mg_{0.063}]$ $[Mg_{0.376}Fe^{2+}_{0.014}Fe^{3+}_{0.136}Al_{1.474}]$ $[Si_{3.87}Al_{0.13}]$ $O_{10}(OH)_2 \cdot nH_2O$ with the cation exchange capability (CEC) of 136.5 mmol/100 g.

2.3 Adsorption of bacterium onto montmorillonite

Batch experiments were conducted to measure *R. Palustris* adsorption onto montmorillonite as a function of initial bacterial quantity, pH, temperature and ionic strength. Different quantities (1.0×10^8, 5.0×10^8, 10.0×10^8, 15.0×10^8, 20.0×10^8 and 25.0×10^8 colony-forming units (cfu) ml^{-1}) of bacterial suspension were added to 10-ml centrifuge tubes containing 50 milligrams montmorillonite. DDI water was supplemented to bring the final volume to 10 ml. Adsorption was conducted at various pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) in which 50 mg of montmorillonite was added to 10 ml of bacterial suspension containing a cell concentration of 15.0×10^8 cfu ml^{-1}. A similar experiment was carried out in the temperature range of 10^oC to 50^oC. The ionic strength experiment was performed by suspending the bacteria in DDI water in the presence of 0.1-100 mmol L^{-1} of Na^+ ($NaNO_3$, Sigma). In each experiment, the bacteria-mineral mixture was allowed to shake (200 rpm) for 2 h at 30^oC except when measuring the effect of incubation temperature on *R. Palustris* adsorption on montmorillonite.

Separation of the unattached bacteria from the fraction containing mineral powder and attached bacteria was accomplished by injecting a sucrose solution (60% by wt.) into the bottom of the mineral-bacteria suspension (Yee *et al*., 2000). Because sucrose is denser than the bacteria suspended in solution but is less dense than the mineral grains (including those with bacteria attached), the sucrose creates a density gradient. The mineral powder, with any adsorbed bacteria, sinks to the bottom of the test tube, and the unadsorbed bacteria and aqueous solution float on top of the sucrose layer. After the sucrose separation, the unattached bacterial fraction was extracted with a pipette and counted using the double-plate method. Control experiments were performed without the mineral present to determine whether adsorption to the test tubes occurs and to quantify the efficiency of the separation technique. The percentage of attached bacteria was determined as follows: (Initial bacterial quantity - Actual quantity of unattached bacteria)/Initial bacterial quantity. The actual quantity of unattached bacteria was measured by adding the amount of the unattached bacteria and the loss in the separation procedure (control). All experiments were done in triplicate.

2.4 Interaction of bacterium with montmorillonite

Sterile tubes containing appropriate malate-basal medium were inoculated anaerobically with *R. Palustris* for 72 h at 30^oC. Then, corresponding weight samples of montmorillonite were weighed and placed directly into sterile test tubes. Tubes containing *R. Palustris* without any clay materials were treated as control. All tubes were incubated anaerobically at 30^oC for 6 h and then the sample solutions, filtered using 0.45μm Millipore membranes, were prepared to determine the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) using a semi-automatic biochemical

analyzer (Technicon, RA-XT, USA) with the test kit (Abbott Laboratory, Chicago, IL, USA), according to manufacturer's instructions. Enzyme activities were expressed as specific activity (U L^{-1}) and all experiments were performed in triplicate.

Cell surface hydrophobicity of *R. Palustris* was determined using an assay measuring bacterial adherence to hydrocarbons, which is based on the partitioning of cells in a two-phase system (Rosenberg *et al*., 1980; Zhang and Miller, 1994). In this study, the hydrocarbons used were dimethylbenzene, which were added to a subsample of the culture and mixed according to Wang and Han (2007). The cells were washed twice to remove any interfering solutes and then resuspended in a buffered salt solution (pH 7.0) containing Na_2HPO_4 and NaH_2PO_4 (0.2 mol·L^{-1}; Sangon, China) to a final density of 10^8 cfu mL^{-1}. Then, the montmorillonite with a predetermined concentration was weighed and supplemented with the previously mentioned bacteria. Afterwards, the bacteria-mineral mixture was allowed to shake (200 rpm) for 2 h at 30°C. Tubes containing *R. Palustris* without any clay materials were treated as the control. Finally, the previously mentioned bacterial cells of the bacteria-mineral mixture (4.0 mL) and dimethylbenzene (2.0 mL) were vortexed in a screw-top tube (10 mL) for 3 min. Then, the dimethylbenzene and aqueous phases were allowed to separate for 30 min. The aqueous phase was recovered with a Pasteur pipette and the turbidity, at 600 nm, was measured. The percentage adhesion was calculated as follows: 100 × (OD_{600} before mixing - OD_{600} after mixing) /OD_{600} before mixing, where OD_{600} is the optical density at 600 nm. All experiments were also performed in triplicate.

2.5 Statistical analyses

Analysis of variance (One-way ANOVA) and the Student's *t*-test were used to determine the significant ($P < 0.05$) differences between the tested groups. The Kruskal-Wallis test was used before the ANOVA analysis. All statistics were performed using SPSS for Windows version 11.5 (SPSS, Chicago, USA).

3. Results

3.1 Effect of initial bacterial quantity on adsorption

Fig. 1 showed the results of the initial bacterial quantities on *R. Palustris* adsorption onto the montmorillonite. The percentage of bacteria adsorbed ranged from 61.07 ± 5.05% to 77.57 ± 4.47%. The highest adsorbed percentage was determined when the initial bacterial quantity was 15.0×10^8 cfu ml^{-1}, which was significantly different ($P < 0.05$) compared with the other quantities used (1.0×10^8, 5.0×10^8 and 10.0×10^8 cfu ml^{-1}). However, no significant decrease in adsorption was observed above 15.0×10^8 cfu ml^{-1}. The numbers of actual bacterial quantities at various initial concentrations were counted. As shown in Table 1, the actual quantity of adsorbed cells was significantly correlated with the initial quantities of *R. Palustris* ($R^2 = 0.9875$, $P < 0.05$, n = 6). Furthermore, the remarkable increase in the number of bacterial cells adsorbed by montmorillonite was observed below the initial cells quantity of 20.0×10^8 cfu ml^{-1}.

Table 1. Effect of initial bacterial quantities on the actual bacterial, *R. Palustris*, quantity after adsorption onto montmorillonite. Results are presented as means ± S.D. of triplicate observations.

Initial quantity ($\times10^8$ cfu ml^{-1})	Actual quantity ($\times10^8$ cfu ml^{-1})
1.00	0.61 ± 0.05
5.00	3.19 ± 0.29
10.00	6.62 ± 0.53
15.00	11.64 ± 0.67
20.00	15.34 ± 0.97
25.00	17.13 ± 1.32

Table 2. Effect of montmorillonite on *R. Palustris* enzyme activities (U L^{-1}) of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH). Results are presented as means ± S.D. of triplicate observations. Means in the same line with different superscripts are significantly different ($P < 0.05$).

Indexes/Groups	Control	Treatment
AST	10.41 ± 1.97^a	10.55 ± 1.98^a
ALT	7.79 ± 1.19^a	7.93 ± 1.70^a
LDH	12.24 ± 1.58^a	12.46 ± 1.86^a

3.2 Effect of pH on adsorption

The cell adsorption on montmorillonite was significantly affected by pH and decreased gradually from pH 4.0 to 10.0 (Fig. 2). The remarkable decrease in cell adsorption was observed above pH 8.0. The data also revealed that the adsorption percentage at pH 4.0 (80.47 ± 7.28%) was significantly different ($P < 0.05$) compared with that at pH 10.0 (54.83 ± 7.11%).

3.3 Effect of temperature on adsorption

The effect of temperature on the adsorption of bacterial cells on montmorillonite is illustrated in Fig. 3. Cell adsorption increased from 10°C to 35°C and then decreased. The temperature under which the maximum amount of adsorption for montmorillonite was

reached was 35°C. The percentage of *R. Palustris* adsorption on montmorillonite at 35°C was 78.07 ± 6.25%, while only 37.87 ± 6.45% of bacterial cells were adsorbed at 50°C. In addition, significantly lower ($P < 0.05$) bacterial adsorption was found at 10°C (43.83 ± 4.55%) than that at 35°C. However, there were no significant differences ($P > 0.05$) among the treatments at 30°C, 35°C and 40°C.

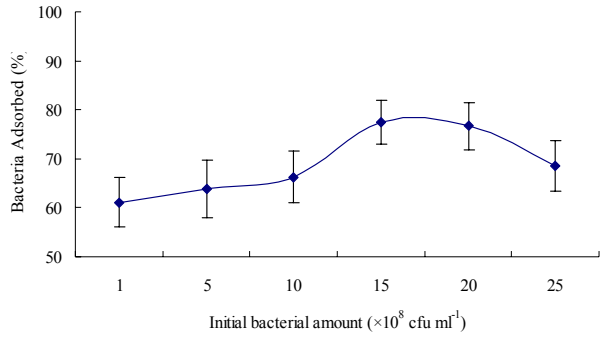

Fig. 1. Effect of initial bacterial quantity (1.0×10^8, 5.0×10^8, 10.0×10^8, 15.0×10^8, 20.0×10^8 and 25.0×10^8 cfu ml^{-1}) on *R. Palustris* adsorption onto montmorillonite.

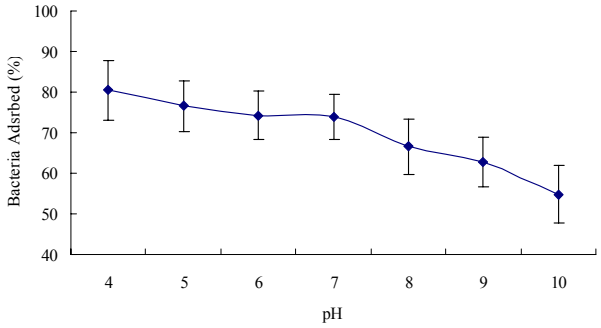

Fig. 2. The pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) dependence of bacterial, *R. Palustris*, adsorption onto the montmorillonite surface.

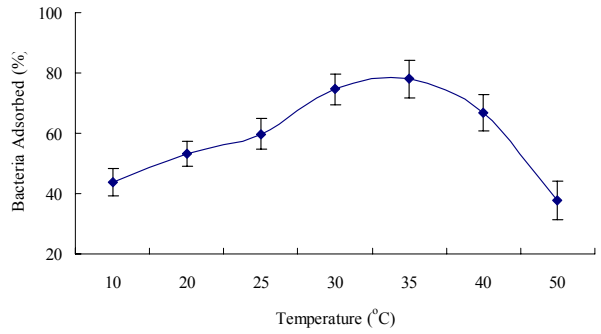

Fig. 3. Effect of temperature (10 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C and 50 °C) on bacterial, *R. Palustris*, adsorption onto the montmorillonite surface.

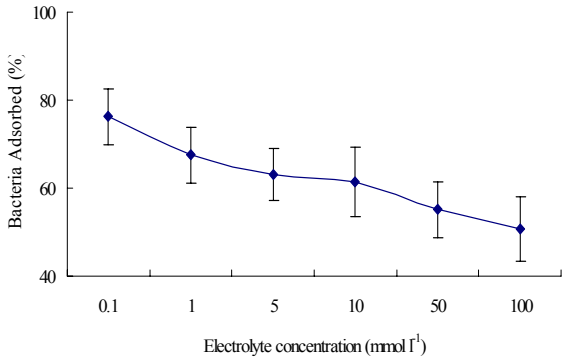

Fig. 4. Ionic strength (0.1-100 mmol L^{-1} of Na^+) experiment of bacterial, *R. Palustris*, adsorption onto montmorillonite.

3.4 Effect of ionic strength on adsorption

As shown in Fig. 4, the change obtained in the ionic strength (0.1-100 mmol L^{-1} of Na^+) experiment for bacteria (*R. Palustris*) adsorption onto montmorillonite were similar to the effect observed for adsorption on pH. The bacteria adsorbed percentage was decreased from 76.20 ± 6.44% to 50.73 ± 7.36% with increased ionic strength (0.1 mmol L^{-1} of Na^+ and 100 mmol L^{-1} of Na^+, respectively).

3.5 Effect of montmorillonite on bacterial enzyme activity and cell hydrophobicity

The effect of mineral montmorillonite on *R. Palustris* enzyme activities of AST, ALT and LDH is shown in Table 2. The bacteria treated with the montmorillonite displayed higher enzyme activities of AST, ALT and LDH compared to those treated without any clay materials. However, there were no significant differences ($P > 0.05$) in these enzyme activities between the treatment and control groups.

The effect of mineral montmorillonite on bacterial cell hydrophobicity of *R. Palustris* is presented in Fig. 5. Compared with the control (48.43 ± 6.10%), montmorillonite supplementation significantly increased ($P < 0.05$) cell surface hydrophobicity of *R. Palustris* (69.93 ± 6.92).

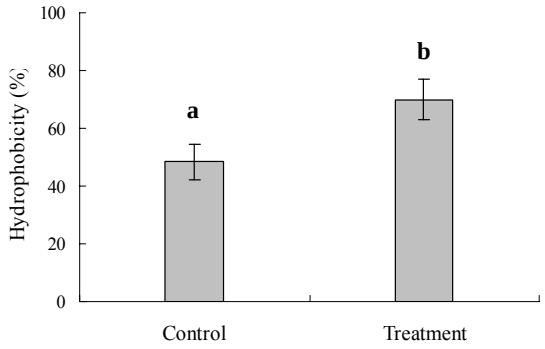

Fig. 5. Effect of montmorillonite on bacterial cell hydrophobicity (%) of *R. Palustris*. Means with different letters are significantly different ($P < 0.05$).

4. Discussions

Adhesion of bacteria onto solid surfaces is a necessary event in nature for the utilization of inorganic and organic values and for the enhanced growth of bacteria (Deo *et al*., 2001). To our knowledge, this is the first study presenting bacteria of *R. Palustris* adsorption on montmorillonite clay. Although a higher adsorption percentage was observed when the initial bacterial quantity was 15.0×10^8 cfu ml^{-1}, the actual quantity of adsorbed bacterial cells increased with continuously increasing initial concentrations of *R. Palustris*. This observation indicated that increased bacterial cell quantities could improve the interaction between *R. Palustris* and montmorillonite. Alternatively, the data demonstrated that the adsorption reaction might be connected with the nature of the surface of the mineral as well.

Montmorillonite is a subset of aluminosilicate clay that possesses a 2:1 layer structure (Borchardt, 1989). Its surface element has various dissociations at different pH values and a range of behaviors in different surface capabilities (Tertre *et al*., 2006). In our study, as the pH increased, the percentage of bacterial adsorption on montmorillonite decreased. The forces governing bacterial adsorption on mineral surfaces include electrostatic and non-electrostatic interactions. The electrostatic force originates from the Coulombic interaction between two charged entities, whereas the non-electrostatic ones come from the hydrogen bonding, van der Waals force, hydrophobic interactions and so forth (Deo *et al*., 2001; Rong *et al*., 2008). Therefore, the various bacterial cell adsorption percentages at different pH values were associated with the interaction of these forces, similar to the results reported for the interaction between bacteria and minerals (Yee *et al*., 2000; Shashikala and Raichur, 2002).

The extent of bacterial adsorption on solid surfaces is affected by the bacterial physiological state, and vigorous bacterial metabolism facilitates their adsorption abilities (Pethica, 1980). In the present study, a greater extent of *R. Palustris* adsorption onto the montmorillonite was observed in the range of temperatures from 30°C to 40°C. Thus, this greater adsorption might be associated with the physiological state of *R. Palustris* since its activity was optimum in this temperature range. A similar finding was obtained by Jiang *et al*. (2007), who investigated the effect of temperature on *P. putida* adsorption onto clay minerals and showed that the temperatures under which the maximum amount of adsorption was reached were 15, 25 and 35°C for goethite, kaolinite and montmorillonite, respectively.

In the present study, the results also showed that as the concentration of ionic strength increased, the percentage of adsorbed bacterial cells decreased. A previous study showed that the adsorption of *B. subtilis* onto corundum decreased significantly with increasing ionic strength (Yee *et al*., 2000). The theory of electrolyte effects on interparticle interaction is referred to as the D.L.V.O. theory (Derjagiun and Laudau, 1941). In suspension, a potential exists between charged particles and the bulk medium. As a result, counter-ions are attracted to the surface to form a double layer of charges. At low ionic strength, the double layers associated with both surfaces of montmorillonite were relatively thick, and the attractive electric fields extended further into solution. This effect increased the potential for adsorption. As the ionic strength increased, the higher concentration of electrolyte ions limited the interaction between the two surfaces and, though they remain oppositely charged, adsorption was reduced. This finding was not in agreement with previous studies showing that the increase in bacterial adsorption on minerals with increasing cation concentration occurs below 100 mmol L^{-1} (Gordon and Millero, 1984; Jiang *et al*., 2007). This difference in adsorption could be attributed to the species and surface characteristics of the experimental bacteria used in the present work in contrast to their study.

AST, ALT and LDH are all intracellular enzymes and are generally treated as indicators of bacterial injury (Korzeniewski and Callewaert, 1983). Under physiological conditions, little or no intracellular enzymes are released from the cell, except for structural injury to the cell wall and/or a change in the permeability of the cell membrane (Guo *et al*., 2005). Therefore, the data obtained for the intracellular enzyme activities of bacteria treated with montmorillonite suggested that the permeability of the cell membranes did not significantly change and the bacteria did not suffer injury.

Microbial cell surface hydrophobicity is recognized as one of the determining factors in microbial adhesion to bioremediation surfaces (van Loosdrecht *et al*., 1987a; 1987b), which is a phenomenon observed commonly in natural and engineered systems (Rosenberg and Doyle, 1990; Wang *et al*., 2005). The cell wall hydrophobicity of *Bacillus sp*. was studied on the basis of the amount of bacteria present in a hydrocarbon/water two-phase system. The results showed that the bacteria with higher levels of cell wall hydrophobicity have a better bioremediation capability (Wang and Han, 2007). In the present study, higher ($P < 0.05$) cell surface hydrophobicity was observed for bacteria supplemented with montmorillonite compared with the untreated bacteria. This observation suggested that the *R. Palustris* attached to montmorillonite clay might have easier access to the soluble materials and organic matter as a source of nutrition and thus had a greater impact on environmental bioremediation.

In conclusion, our results indicate that the initial bacterial quantity, pH, temperature and ionic strength were all important in controlling the adsorption of *R. Palustris* onto montmorillonite clay. No significant differences in enzymes activities of AST, ALT and LDH were observed. However, higher cell surface hydrophobicity was observed in the bacteria supplemented with montmorillonite. The adhesion process is controlled by physicochemical interactions between cells and mineral substrates. Although this study employed a single type of bacterium, similar mechanisms may be important in determining the distribution of *R. Palustris* in the environment. Further studies using other techniques are needed to investigate the interactions of *R. Palustris* with various minerals and soil components in different environmental conditions. In addition, the bioremediation capability of *R. Palustris* supplemented with montmorillonite will be evaluated in future studies.

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