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Impact of the bacterium *Pseudomonas fluorescens* and its genetic derivatives on vermiculite: Effects on trace metals contents and clay mineralogical properties

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ABSTRACT

Using bacteria of the strain *Pseudomonas fluorescens* wild type CHA0 and its genetic derivative strains CHA631, CHA77, CHA89, CHA400, CHA661 (which differ in one gene only) the changes in chemical, mineralogical and rheological properties of the clay mineral vermiculite affected by microbial activity were studied in order to test whether the individually different production of metabolites by the genetically engineered strains may alter the clay mineral vermiculite in distinct ways. With the novel strategy of working with living wild type bacteria, their genetic derivatives and clay, the following properties of the mineral altered by the various strains of *Pseudomonas fluorescens* were determined: grain size, X-Ray diffraction pattern, intercrystalline swelling with glycerol, layer charge, CEC, BET surface and uptake of trace elements. All experiments were carried out with suspensions containing the bacteria in the nutrient medium and abiotic control suspensions with the nutrient medium only. The wild type CHA0 and strains CHA631, CHA77, CHA89 caused a decrease in the apparent grain size whereas strains CHA400 and CHA661 caused an increase in apparent grain size due to aggregation. Aggregation was furthermore evidenced by the smaller BET (Brunauer–Emmett–Teller) surface. In the absence of bacteria the clay mineral incorporates Na^+ from the medium. In contrast this is inhibited by the wild type CHA0 and the genetic derivatives CHA631, CHA77, CHA89 whereas strains CHA400 and CHA661 allowed the exchange of K^+ and Mg^{2+} against Na^+ in vermiculite. Among all analyzed the trace elements, Fe, Mn and Cu are the most interesting. Fe and Mn are taken up from the clay mineral by all bacterial strains whereas Cu is only removed from vermiculite by strains CHA0, CHA77, CHA400, CHA661. The latter mentioned strains all produce 2,4-diacetylphloroglucinol and monoacetylphloroglucinol which can complex Cu efficiently. Therefore the alteration of only one gene of the bacteria is causing significant effects on the clay mineral.

1. Introduction

Bacteria are ubiquitous in natural soil, subsoil and rocks and are in permanent contact with clay minerals (Neher and Rohrer, 1959; Stotzky, 1986; Mahmood and Rama Rao, 1993; Zeyer, 1993). Concern about the microbial degradation of clay minerals (Filip, 1979; Dietz and Mörtel, 1987; Mörtel and Schmelzer, 1993) has been raised due to the use of clays as waste deposit barriers (McKinley et al., 1985; Madsen, 1998; Madsen, 1999; Pedersen et al., 2000). Moreover, clay minerals in conjunction with bacteria are known to play a major role in preventing dispersion of potential contaminants such as trace metals and organic pollutants (e. g. Eckhardt, 1985; Kretzschmar and Voegelin, 2001; Brantley, 2002; Fowle and Fein, 2002). Beyond this, bacteria are able to sequester trace elements in their cell wall and to release them again (e. g. Beveridge and Fyfe, 1985; Brantley et al., 2001). Clay minerals form a reservoir for plant nutrients and for macronutrients for bacteria like K, Na, Ca, Mg, Al and NH_4 which constitute the bulk of the cell walls. Trace element micronutrients like V, Mn, Fe, Co, Ni, Cu or Zn are widely found in proteins. Even tough uptake of metals and trace elements by microbes have been recorded in many scientific publications (e. g. Beveridge and Fyfe, 1985), very little is known about the uptake of metals and trace elements by microbes from clay minerals. Due to their low concentrations in most environments, metals and trace elements require organic ligands for their transport. Among the metals, Fe is the only bioessential element known to require a specific organic ligand for transport (e. g. Neilands, 1989). These specific organic ligands called siderophores are secreted by many organisms for sequestration of Fe under neutral, aerobic conditions. Many of these siderophores can chelate other bioessential nutrients than Fe as well (e. g. del Olmo et al., 2003).

So far most investigations have focused on reduction of Fe(III) and the dissolution of various clay minerals by bacteria (Stucki et al., 1992; Kostka et al., 1996; Gates et al., 1998). These experiments were carried out by using wild type bacteria or more often by using chemically synthezised metabolites of the bacteria only. Hence, our novel strategy

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was the use of living wild type bacteria, their genetic derivatives and clay in order to determine the changes in chemical and mineralogical properties of clay minerals affected by microbial activity.

In a first article (Müller and Défago, 2006), we investigated the alteration of vermiculite due to the interaction with the soil bacterium *Pseudomonas fluorescens* strain CHA0. This strain is well known to protect tobacco against black root rot in vermiculitic but not illitic soil and to survive up to six months in vermiculite suspensions (whereas the bacteria die in illite suspensions after only one month, see e. g. Stutz et al., 1989). Moreover, numerous mutants of strain CHA0 are readily available and a close relative of strain CHA0 (Pf5) is already fully sequenced (Haas and Défago, 2005).

For the present work the well-characterized strain CHA0 was chosen, as several genetic constructs are easily available. In order to elucidate the type of physico-chemical interactions, genetic derivatives of strain CHA0 were taken. The genetic derivatives (in particular strains CHA631, CHA77, CHA89, CHA400 and CHA661) used in this study differ from the wild type strain CHA0 through the absence, synthesis or the overproduction of metabolites involved in disease suppression (see Table 1). The phenotype of these constructs is stable during several weeks of bacterial growth.

Our objective was to study the interactions between the clay mineral vermiculite and the bacterium *P. fluorescens* CHA0 including its genetic derivatives in order to detect differences in alteration of vermiculite caused by the various bacterial strains.

The genetic derivatives (in particular strains CHA631, CHA77, CHA89, CHA400 and CHA661) used in this study differ from the wild type strain CHA0 through the absence, synthesis or the overproduction of metabolites involved in disease suppression (see Table 1).

As a first step the experiments were done in an artificial way with suspensions; further experiments will approach a more natural system. The interactions studied included the changes in chemical (analysis of trace elements), mineralogical (analysis via X-ray diffraction pattern, grain size distribution, intracrystalline swelling, cation exchange capacity, layer charge, specific surface) and mechanical properties of clay minerals affected by microbial activity.

Table 1
Pseudomonas fluorescens strain CHA0 and its genetic derivatives modified in the production of metabolites.

Strain	Relevant genotype	Relevant phenotype	Reference
CHA0	Wildtype	2,4-diacetylphloroglucinol Monoacetylphloroglucinol Cyanide Pyoluteorin Pyochelin Pyoverdin Protease Salicylic acid	Voisard et al. (1994)
CHA77	Δ*hcnABC*	Cyanide negative Rest see wildtype	Laville et al. (1998)
CHA89	*gacA*::W-km	2,4-diacetylphloroglucinol negative Monoacetylphloroglucinol negative Cyanide negative Pyoluteorin negative Protease negative Pyochelin overproducer Salicylic acid overproducer	Heeb and Haas (2001)
CHA400	pltC::Tn5	Pyoverdin negative Rest see wildtype	Keel et al. (1989)
CHA631	Δ*phlA*	2,4-diacetylphloroglucinol negative Monoacetylphloroglucinol negative Rest see wildtype	Schnider-Keel et al. (2000)
CHA661	pltC::Tn5	Pyoluteorin negative Rest see wildtype	Maurhofer et al. (1994)

2. Materials and methods

Vermiculite supplied by Thermex, Austria, was used for all the experiments. Prior to use, the vermiculite was expanded by heating with conc. H_2O_2 (30%) as described in Stutz et al. (1989). Later it was crushed with a mixer and afterwards oven (60 °C) or air dried. The dry vermiculite was ground in a mortar.

The following methods were used to determine the chemical, mineralogical and mechanical changes in vermiculite due to microbial activity: The grain size analysis was carried out with the light scattering apparatus MICROTRAC FRA (Leeds & Northrup) on the basis of direct scattering of laser light in the range 1–60° on particles in a suspension.

X-ray diffraction measurements were made using a Bragg–Brentano diffractometer (Bruker AXS D8 CuKα with automatic divergence slit, graphite monochromator and sample spinner) in the range of 1.5 to 65° 2θ with a step width of 0.02° 2θ and a counting time of 3 s. Oriented samples were treated with glycerol to identify characteristic swelling. The relationship between XRD basal spacings and the mean layer charges calculated from the n-alkylammonium ion-exchange technique (Lagaly and Weiss, 1969) were used to estimate the mean total layer charge (Olis et al., 1990).

The cation exchange capacity was determined using the exchange method with ammonium acetate (MacKenzie, 1951). See also Meier and Kahr (1999).

The external specific surface was determined with the multipoint N_2 adsorption technique (Gemini III, Micromitics) and interpreted with the BET-method (Brunauer et al., 1938).

Environmental Scanning Electron Microscopy (ESEM) analysis was performed in order to detect differences in the microstructural morphology between the blank samples and the samples containing bacteria. Wet samples taken directly from the batch suspensions were analyzed using a FEI Quanta 600 ESEM.

For the analysis of trace elements by Laser Ablation ICP-MS fused glasses of the vermiculite samples were prepared by mixing a 5:1 ratio of flux to the finely ground samples. The LA ICP-MS analyses of major, minor and trace elements were performed using a pulsed 193 nm ArF Excimer laser (Lambda Physik, Germany) with a homogeneous beam delivery prototype system similar to a Geolas system (Microlas, Germany, see Günther et al., 1997) in combination with an ELAN 6100 DRC (Perkin Elmer, Canada) ICP-MS. The samples were loaded along with the glass reference standards NIST 610 in a 15 cm^3 ablation cell and put on the stage of a modified petrographic microscope. To the laser ablation carrier gas, helium, the make up gas argon was admixed after the ablation cell and the aerosole carried to ICP-MS. The linear dynamic range of about 9 orders of magnitude in dual detector mode could be achieved by dual detector calibration. Analyses were performed in sequence and every ablation was stored individually as a transient signal. 230 readings were measured per replicate. Data reduction for concentration and limit of detection calculation were undertaken using the software LAMTRACE and in-house spreadsheets following methods as described by Longerich et al. (1996) and Heinrich et al. (2003). Aluminium was used as an internal standard (average of 15 analysis of Al in vermiculite as published by Jasmund and Lagaly (1993) whereas NIST 610 (Perkins et al., 1997) and GSE 1G (Guillong et al., 2005; Jochum et al., 2007) were used as an external standard. The following trace elements were analyzed: Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Rb, Sr, Y, Zr, Nb, Mo, Ag, Cd, Sn, Sb, Cs, La, Ce, W, Tl, Pb, Bi, U.

For all the batch experiments the following procedure was established:

For run 1, strain CHA0 and strain CHA631 (VB1 = abiotic control, CHA0 = slurry containing the wild type strain and CHA631 = slurry