**Low-temperature feldspar and illite formation through bioreduction of
Fe(III)-bearing smectite by an alkaliphilic bacterium**

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Further revised for Chemical Geology

April 24, 2015

ABSTRACT

Biogenic mineral assemblages that form from circumneutral microbial reduction of iron in smectite have been suggested as biosignatures in the geological record. However, mineralogical transformation of smectite mediated by microbes under extreme pH condition is still poorly known. The objective of this study was to understand the reduction capacity of structural Fe(III) in iron-rich smectite (nontronite, NAu-2) by a novel anaerobic alkaliphile (strain CCSD-1) isolated from the deep subsurface, and associated mineralogical changes. The experiments with CCSD-1 were conducted in a growth medium containing the electron shuttle anthraquinone-2,6-disulfonate (AQDS) at pH 9.4. The Fe(II) concentration was monitored over the course of the experiment via wet chemistry, and unreduced and reduced nontronites were characterized with X-ray diffraction (XRD), and scanning and transmission electron microscopy (SEM and TEM). The results indicate that strain CCSD-1 utilizes proteinaceous substrates (yeast extract and tryptone) to reduce structural Fe(III) in smectite with the maximum reduction extent of 26.2%. Mineralogical analysis confirmed that biogenic plagioclase (Na-Ca feldspar) and illite formed after bioreduction. Our work shows that the interaction between alkaliphile and iron-bearing smectite could account for low-temperature feldspar and illite formation and these minerals may be used as biosignatures in sedimentary rocks.

Keywords: iron reduction; smectite illitization; authigenic feldspar; deep subsurface

1. Introduction

Microorganisms are important agents in catalyzing surficial geochemical reactions, such as mineral formation, rock decomposition (weathering), elemental redox cycles, and other geological processes (Falkowski et al., 2008; Gadd et al., 2010). Among these biogeochemical reactions, the field of microbe-mineral interaction has attracted much attention in the last two decades (Banfield and Nealson, 1997; Dong, 2010; Gadd et al., 2010). Microorganisms play a passive or active role in (trans)formation of various minerals (e.g., oxides, carbonates, silicates, phosphates and others) (Banfield and Nealson, 1997; Konhauser and Urrutia, 1999; Dong et al., 2009; Gadd et al., 2010). In comparison to chemically-formed minerals, biogenic minerals may possess unique chemical, structural, or morphological signatures (Fortin, 2004; Javaux and Benzerara, 2009). Therefore, it is believed that biogenic minerals, especially those that form at ambient temperature, can be used as biosignatures to infer past life activities on Earth (Fortin and Langley, 2005; Chan et al., 2011; Li et al., 2011; Crosby et al., 2014) and beyond (Pósfai et al., 1998; Banfield et al., 2001; Thomas-Keprta et al., 2000). Dolomite is a well-studied example. Dolomite is common in sedimentary record, yet efforts to synthesize dolomite at low temperatures (<80°C) have resulted in little progress. Mounting evidence has suggested that certain types of microbes can overcome the kinetic energy barrier and promote dolomite formation under room temperature laboratory conditions (Mckenzie and Vasconcelos, 2009, and references therein). Notably, globule-shaped dolomite observed in microbially catalyzed bench-scale systems has been proposed as a characteristic

1 biosignature for ancient dolomite in the geological record (Sánchez-Román et al.,
2 2008; Mckenzie and Vasconcelos, 2009, and references therein).

3 Formation of low-temperature illite through the smectite-to-illite (S-I) reaction is
4 another example showing how microbes challenge our traditional concepts of mineral
5 transformation. The S-I reaction (also termed smectite illitization) is one of the
6 important reactions in mudstones and shales, and accounts for abundant authigenic
7 illite in sedimentary rocks (Dong et al., 1997; Pevear, 1999). During sediment
8 diagenesis, the S-I reaction proceeds through mixed-layer illite-smectite (I-S)
9 intermediates, including smectite-rich R0, ordered R1, R2, R3 I-S (R, Reichweite
10 ordering parameter) and end-member illite (Pevear, 1999). It was believed that the S-I
11 reaction is an abiotic process that typically requires conditions of 300-350 °C, 100
12 MPa, and 4-5 months to occur. Therefore, the increased value of %I in I-S had been
13 commonly used as a paleo-thermometer to constrain the thermal history of a
14 sedimentary basin (Hoffman and Hower, 1979; Pevear, 1999).

15 However, Kim et al. (2004) provided compelling evidence for the first time
16 revealing that a dissimilatory iron-reducing bacterium (DIRB), *Shewanella oneidensis*
17 MR-1, is capable of facilitating smectite illitization at room temperature and one
18 atmosphere within two weeks. This S-I reaction was interpreted to occur as a result of
19 microbial reduction of structurally-coordinated Fe(III) in smectite by DIRB, reductive
20 dissolution of smectite, and subsequent formation of illite (Kim et al., 2004). A
21 growing body of work has subsequently suggested that a wide variety of DIRB (e.g.,
22 *Shewanella* strains: Zhang et al., 2007a; Gaines et al., 2009; Jaisi et al., 2011; Koo et

1 al., 2014; Liu et al., 2014; *Thermoanaerobacter ethanolicus*: Zhang et al., 2006,
2 2007b; *Thermus scotoductus*: Jaisi et al., 2011) and sulfate-reducing bacteria (SRB)
3 (Liu et al., 2012) catalyze illite formation via a similar mechanism. In addition to illite,
4 some other secondary minerals have also been observed as a result of bioreduction of
5 iron-bearing smectite, such as amorphous silica globules (Dong et al., 2003; O'Reilly
6 et al., 2005; Zhang et al., 2007b; Liu et al., 2011, 2014), high charge smectite with
7 increased Al/Si ratios (O'Reilly et al., 2005; Zhang et al., 2007a; Liu et al., 2011), and
8 other various byproducts (e.g., vivianite, siderite, calcite, and iron sulfide particle),
9 depending on medium and buffer type (Li et al., 2004; Dong et al., 2009; Jaisi et al.,
10 2011). Upon burial diagenesis or low-grade metamorphism, silica, high charge
11 smectite, and iron sulfide could be transformed to more stable phases (quartz, illite,
12 and pyrite). Consequently, certain mineral assemblages, such as low-temperature illite,
13 quartz, calcite, and pyrite, may be used as biosignatures for the presence and activities
14 of iron-reducing microorganisms in geological time. Several studies have successfully
15 used this type of mineral assemblages to identify the role of iron reducing bacteria in
16 sedimentary systems (Sanz-Montero et al., 2009; Vorhies and Gaines, 2009). However,
17 to date most studies on clay-microbe interactions have been conducted at near-neutral
18 pH, and the impact of alkaliphilic bacteria on smectite transformation remains poorly
19 understood.

20 Alkaliphilic microorganisms, those that grow at pH in excess of 9, are widely
21 distributed in almost all environments, particularly in soda lakes and alkaline deserts
22 (Horikoshi, 1999). To date, several species of anaerobic alkaliphiles have been

1 isolated and they can respire solid-phase iron oxides (e.g., Ye et al., 2004, Zhilina et
2 al., 2009). However, it is unclear whether alkaliphilic bacteria can use structural iron
3 in phyllosilicates, and if so, how secondary mineral assemblages differ from those
4 formed in circumneutral environments. Even though naturally-occurring alkaline
5 environments are not common on modern Earth, it is postulated that alkaline
6 conditions may have predominated in large areas of the Precambrian ocean (termed
7 soda ocean) (Kempe and Degens, 1985; Kempe et al., 1989), and even in the
8 subsurface of Europa (Kempe and Kazmierczak, 2002). Because clay minerals are the
9 most abundant mineral constituents of marine sediments and one of the main Fe(III)
10 pools in sedimentary environments (Favre et al., 2006; Dong et al., 2009), they can
11 co-exist with alkaliphilic iron-reducing microorganisms. Therefore, understanding the
12 interaction process between clay minerals and alkaliphiles could be essential to assess
13 the possibility of life on early Earth and other planets.

14 In the present study, one novel anaerobic alkaliphile, recently isolated from the
15 borehole of the Chinese Continental Scientific Drilling project (CCSD), was selected
16 to test whether unique byproducts can be formed during microbially-mediated
17 smectite reduction.

18

19 **2. Materials and Methods**

20 **2.1. Site description**

21 The CCSD drilling site is located in Donghai County, Lianyungang City, Jiangsu
22 Province, at the eastern part of the Dabie-Sulu ultra-high pressure (UHP)

1 metamorphic belt (Zhang et al., 2007b). The CCSD project started in June 2001 and
2 ended in January 2005, with the main aim to reconstruct the formation and evolution
3 of UHP metamorphic terrain in eastern China (Xu et al., 2004). The main drillhole of
4 the CCSD project reached a depth of 5518 m. According to previous studies, the UHP
5 index mineral coesite was found in many sections of the main hole, strongly
6 suggesting that the crystalline basement in this area was subjected to UHP
7 metamorphism (Xu et al., 2004).

9 **2.2. Bacterial enrichment and isolation**

10 A drilling mud sample recovered from ca.880-m depth of the CCSD main hole
11 was enriched for microbial growth in the presence of additional carbohydrates. Given
12 the in-situ high pH of the sample (pH≈9.4), the following basal medium for anaerobic
13 alkaliphiles was used (g/L): (NH₄)₂SO₄, 1; NH₄Cl, 0.4; Na₂S₂O₃, 0.1; K₂HPO₄, 0.5;
14 MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.05; NaCl, 10; tryptone 0.25; yeast extract, 0.25;
15 trace element DSMZ 141 (Deutsche Sammlung von Mikroorganismen und
16 Zellkulturen, Braunschweig, Germany) solution, 10 mL; vitamin DSMZ 141 solution,
17 10 mL; NaHCO₃, 2.2; Na₂CO₃, 2.2. The medium was dispensed into 120-mL serum
18 bottles. After adjusting pH to 9.4 with 1 M NaOH, the bottles were purged with
19 ultra-pure N₂ and sealed with thick butyl rubber stoppers, and capped with aluminum
20 caps. After the bottles were autoclaved, approximately 2 g of the mud sample were
21 added into the bottles in an anaerobic chamber (filled with 98% N₂ and 2% H₂, Coy
22 Laboratory Products, Grass Lake, Michigan). The serum bottles inoculated with the

1 mud sample were incubated in the dark at 35 °C. The growth was monitored by visual
2 observation of cell turbidity and precipitation. When growth was evident, transfer to
3 fresh enrichment bottles was carried out. After five transfers, the primary enrichments
4 were then successively pipetted into fresh media to make serial dilutions. An aliquot
5 of 0.5-mL diluted inoculum was added into a roll tube with agar-solidified medium (2
6 g/L agar) as previously described (Zhang et al., 2006). After approximately 3 weeks
7 of incubation, single colonies were picked and again spread onto agar-solidified
8 medium. This process was repeated until one colony type was observed.

10 **2.3. Growth studies**

11 An isolate, designated strain CCSD-1, was selected as a representative for further
12 experiments. The effects of temperature and pH on cell growth were investigated in
13 batch cultures. Growth was determined by measuring the optical density of the culture
14 with a spectrophotometer at 600 nm.

16 **2.4. Sequence analysis of the 16S rRNA gene**

17 The genomic DNA of strain CCSD-1 was obtained using the FastDNA SPIN Kit
18 for soil (MP Biomedicals, Solon, OH, USA). 16S rRNA gene was amplified using
19 bacterial universal primers (27F and 1492R). Sequencing and phylogenetic analysis of
20 the 16S rRNA gene were performed as previously described (Zhang et al., 2006).

22 **2.5. Mineral preparation**

1 Nontronite N_{Au}-2, an iron-rich smectite from the Uley Graphite Mine in
2 Australia, was purchased from the Source Clays Repository of the Clay Minerals
3 Society (West Lafayette, IN, USA). Bulk N_{Au}-2 was gently ground and saturated in
4 0.5 mM NaCl solution overnight. The 0.02-0.5 μm size fraction of N_{Au}-2 was
5 subsequently collected by centrifugation using Stokes' settling law. Once the size
6 fraction of 0.02-0.5 μm was obtained, excess chloride anion was removed by repeated
7 washing in doubly distilled water and its completed removal was confirmed with
8 AgNO₃. Previous studies have shown that N_{Au}-2 contains 23.40% iron by weight, of
9 which 99.40% is Fe(III), and its structural formula can be expressed as
10 $M_{0.72}(Si_{7.55}Al_{0.16}Fe_{0.29})(Al_{0.34}Fe_{3.54}Mg_{0.05})O_{20}(OH)_4$ (M represents the interlayer cation)
11 (Keeling et al., 2000; Jaisi et al., 2005; Luan et al., 2014).

12

13 **2.6. Bioreduction experiments**

14 Batch experiments were conducted to assess the capacity of strain CCSD-1 to
15 reduce structural Fe(III) in N_{Au}-2. These experiments were carried out under growth
16 conditions, which contained 10 mL basal medium as described above, N_{Au}-2 mineral
17 (final concentration, 5 g/L), anthraquinone-2,6-disulfonate (AQDS, 0.1 mM), and
18 CCSD-1 cells. Briefly, N_{Au}-2 mineral was made into suspension with the basal
19 medium in 25-mL Balch culture tubes. After adjusting pH to 9.4 with 1 M NaOH, the
20 bottles were purged with ultra-pure N₂ and sealed with thick butyl rubber stoppers,
21 and capped with aluminum caps. After the bottles were autoclaved, cells of CCSD-1
22 from an anaerobic stock were added into the bottles to achieve a final concentration of

1 ~4×10⁷ cells/mL. AQDS was added as an electron shuttle in all experiments, because
2 the role of AQDS in enhancing Fe(III) reduction by iron-reducing bacteria has been
3 well documented (e.g., Jaisi et al., 2005). Abiotic controls were identical to the
4 experimental systems except that cells were replaced with an equal amount of
5 medium, and all experiments were performed in duplicate. The experimental tubes
6 were incubated in the dark at 35°C.

7

8 *2.7. Fe(II) analysis and cell counts*

9 To monitor the progress of Fe(III) reduction, the total Fe(II) content was
10 measured at selected time points by the 1,10-phenanthroline assay (Amonette and
11 Templeton, 1998). The 1,10-phenanthroline method is based on total digestion of the
12 mineral with HF-H₂SO₄ and has been demonstrated as an effective approach for
13 measuring total Fe(II) in layer silicates (Amonette and Templeton, 1998; Anastacio et
14 al., 2008).

15 Cell numbers throughout the bioreduction experiments were counted by
16 acridine-orange direct counting (AODC). At least 10 fields were counted for
17 calculation of average cell concentrations at each time point.

18

19 *2.8. X-ray diffraction (XRD)*

20 The original and bioreduced NAu-2 samples were studied with XRD to identify
21 any mineralogical changes as a result of bioreduction. Smear mounts (Moore and
22 Reynolds, 1997) were prepared in an anaerobic chamber under room temperature. To

1 identify illitic layers, air-dried and oriented clay slides were saturated with ethylene
2 glycol vapor overnight at 60°C. The ethylene glycolated samples were run on a
3 Scintag X1 X-ray powder diffractometer using CuK α wavelength, a fixed slit
4 scintillation detector, and power of 14000 W (40 kV, 35 mA). The samples were
5 scanned from 2 to 50° 2 θ stepping at 0.02° with a count time of 2s per step.
6 Qualitative identification of mineral phases was made using the Jade 7 program.

7 To detect any possible formation of illite as a result of microbial reduction and to
8 quantify the relative proportion of smectite, mixed-layer I-S, and illite, XRD patterns
9 for the ethylene glycolated samples (unreduced and reduced) were modeled using the
10 Sybilla program (McCarty et al., 2009).

11

12 **2.9. Scanning and transmission electron microscopy (SEM and TEM)**

13 Mineral-microbe spatial relationships and mineralogical changes after
14 bioreduction were further examined using SEM and TEM. Sample preparation
15 followed previous reports (Dong et al., 2003; Liu et al., 2011). Briefly, for SEM
16 observation, after fixation with 2% paraformaldehyde and 2.5% glutaraldehyde, one
17 droplet of clay-cell suspension was placed onto the surface of a glass cover slip. The
18 sample on the cover slip was sequentially dehydrated using varying proportions of
19 ethanol followed by critical point drying. The cover slip was then mounted onto a
20 SEM stub and coated with Au. For TEM observations, diluted suspensions were
21 pipetted onto 300 mesh copper grids with carbon-coated nitrocellulose membrane.
22 The grids were dried overnight in an anaerobic chamber (filled with 98% N₂ and 2%

H₂).

A Zeiss Supra 35 VP SEM with Genesis 2000 X-ray energy dispersive spectroscopy (SEM/EDS) was employed for observation and analysis. The SEM was operated at an accelerating voltage of 10-15 kV. A short working distance (6-10 mm) and low beam current (30-40 µA) were used to achieve the best image resolution. A JEOL JEM-2100 LaB₆ TEM/STEM with a 200 kV accelerating voltage was used for TEM observations. For mineral identification, selected area electron diffraction (SAED) patterns and EDS spectra were obtained.

3. Results

3.1. Strain CCSD-1 description

Cells of strain CCSD-1 are straight or slightly curved rods, 0.2-0.5 µm wide and 6-15 µm long with possible endospore-like structure (Fig. 1A). Cell viability after 1 year of storage further suggests that CCSD-1 is a spore-forming bacterium. According to the phylogenetic analysis of the 16S rRNA gene sequence, strain CCSD-1 forms a tight cluster with several species of the genus *Anaerobranca* in the Clostridium-Bacillus subphylum of Gram-positive bacteria (Fig. 1B). Within this cluster, the 16S rRNA gene sequence of CCSD-1 is 94.5%, 93% , 92.4% and 91.7% similar to *A. gorrschalkii* strain LBS3, *A. californiensis* strain PAOHA-1, *A. zavarzinii* strain JW/VK-KS5Y, and *A. horikoshii* JW/YL-138, respectively. Therefore, on the basis of the 16S rRNA gene sequence, strain CCSD-1 is a novel species within the genus *Anaerobranca*. Strain CCSD-1 grew within the temperature range of

1 15-42 °C with an optimum at 38 °C (Fig. 1C) and a pH range of 6.8-11.2 with an
2 optimum at 9.4 (Fig. 1D).

3 4 **3.2. Microbial reduction of Fe(III) in NAu-2**

5 In the absence of bacterial cells, the extent of abiotic Fe(III) reduction remained
6 low (<1.35%). CCSD-1 cells significantly enhanced NAu-2 reduction (Fig. 2A). In
7 general, the Fe(III) reduction extent showed a rapid rise within the first 7 d followed
8 by a moderate increase and a final plateau (26.20%). Compared to total Fe(II), there
9 was no appreciable aqueous Fe(II) accumulation within the inoculated experimental
10 bottles (data not shown), suggesting that the produced Fe(II) mostly remained within
11 the NAu-2 structure and was adsorbed onto solid surfaces (e.g., residual NAu-2 and
12 secondary minerals).

13 14 **3.3. Cell growth**

15 Cell growth was observed during the early phase of bioreduction (Fig. 2B).
16 Specifically, CCSD-1 cells rapidly grew during the first 5 d, when active Fe(III)
17 reduction occurred, suggesting that strain CCSD-1 uses Fe(III) in nontronite as a
18 terminal electron acceptor for growth. When Fe(III) reduction stopped, cell growth
19 ceased and cells eventually died (Fig. 2B).

20 21 **3.4. XRD and related modeling**

22 Both the abiotic control and bioreduced samples were examined after 35 d of

1 incubation for mineralogical changes after ethylene glycolation (Fig. 3). Compared to
2 the abiotic control, the 001 peak of the bio-reduced sample was broader and of lower
3 intensity (Fig. 3A), which suggests some structural alteration of N_{Au}-2 upon
4 bio-reduction. A hump in the 8 to 10° 2 θ range showed a mixture of discrete illite, R0,
5 R2 I-S, and nontronite as confirmed with Sybilla modeling (Figures 3B and 3C). In
6 addition to mixed-layer I-S, the bio-reduction of N_{Au}-2 by strain CCSD-1 also
7 resulted in a byproduct with two distinct d values of 6.54 and 3.18 Å, which was
8 identified as plagioclase (Na-Ca feldspar) (Fig. 3A). Some plagioclase peaks were
9 absent, possibly due to preferred orientation resulting from the smear mount method
10 used for XRD analysis. The neoformation of feldspar and illite was further evidenced
11 by SEM and TEM data as described below.

13 3.5. SEM and TEM observations

14 Residual solid phases were collected at the end of bio-reduction experiments for
15 SEM and TEM observations. SEM studies showed that residual smectite particles
16 were irregular in shape, and displayed a low Al/Si weight ratio (i.e., < 0.1, similar to
17 pristine N_{Au}-2) and low amounts of K and Fe (particle a shown in Fig. 4A).
18 Interestingly, some particles with distinctly different morphologies emerged within
19 bio-reduced samples. Specifically, euhedral plagioclase, which had an average
20 diameter of 8 μ m, displayed a pseudo-rhombohedral morphology (particle b shown in
21 Fig. 4A). The plagioclase was identified by EDS showing a high Al/Si ratio and a
22 significant amount of Na and Ca. Imprints of cells and associated exopolysaccharide

(EPS)-like materials were observed on the edge of a plagioclase particle (Fig. 4A), indicating that the surface of CCSD-1 cells might act as a nucleation site for feldspar formation. Furthermore, block-like (particle c shown in Fig. 4B) and spherical (particle d shown in Fig. 4C) particles were also found after bioreduction, which were identified as illite and silica, respectively, on the basis of EDS analysis.

TEM images, EDS, and SAED provided additional evidence for the formation of plagioclase and illite (Fig. 5). The biogenic plagioclase occurred as an euhedral crystal showing a high Al/Si ratio and significant amounts of Na and Ca (Fig. 5A). In contrast to wavy smectite layers with 12 Å, illite fringes were straight and constrained within a well-defined packet with 10 Å d-spacing (Fig. 5B), as consistent with previous observations of illite (Dong et al., 1997).

4. Discussion

4.1. Bioreduction of structural Fe(III) in nontronite by strain CCSD-1

Since the first isolation of DIRB in the 1980's, the process of microbial reduction of Fe(III) in solid phases and associated mineral transformation have been the focus of a great deal of research in the field of geomicrobiology (Melton et al., 2014). In contrast to the vast studies on neutrophilic DIRB (e.g., primarily those from the genera *Shewanella* and *Geobacter*), there is a limited number of reports focusing on DIRB from alkaline environments. The reason is probably the fact that iron reduction is a proton-consuming reaction and therefore iron respiration in alkaline environments is likely energetically less favorable (Bethke et al., 2011; Flynn et al., 2014). However,

1 several species isolated from extreme alkaline environments have been demonstrated
2 to be capable of coupling iron reduction to growth including: *Alkaliphilus*
3 *metalliredigens* QYMF (Ye et al., 2004) and *A. peptidofermentans* Z-7036: (Zhilina et
4 al., 2009); some *Bacillus* species including *Bacillus* sp. SFB (Pollock et al., 2007) and
5 *B. pseudofirmus* MC02 (Ma et al., 2012); and a strain with 98% phylogenetic
6 similarity to *Anaerobranca horikoshii* (Gorlenko et al., 2004). These obligate
7 alkaliphiles can utilize low-molecular-weight organic acids (e.g., lactate and acetate)
8 or proteinaceous substrates (e.g., yeast extract and tryptone) to reduce Fe(III) either in
9 soluble form or solid-phase. In this study, it was shown that strain CCSD-1 likely
10 belonged to *Anaerobranca* that could reduce structural Fe(III) in smectite.
11 Interestingly, strain CCSD-1 and the aforementioned alkaliphilic iron reducers are all
12 members of the Clostridium-Bacillus subphylum, distinctly different from the
13 phylogenetic affiliation of neutrophilic iron reducers (subphylum *Delta*- or
14 *Gammaproteobacteria*). To overcome the proton scarcity in alkaline environment, Na⁺
15 ions may replace protons to create an electron motive force benefiting certain
16 alkaliphiles under saline conditions (Thorpe et al., 2012), which might be the reason
17 for the common occurrence of iron-reducing alkaliphiles in saline environments.

18 As with its closest known isolates (e.g., *A. gorrschalkii* and *A. californiensis*),
19 strain CCSD-1 grows fermentatively on various proteinaceous substrates, such as
20 yeast extract, peptone, and tryptone when coupled with reduction of Fe(III) in
21 smectite (Fig. 2). Actually, the iron reduction capability of fermentators is not limited
22 to *Anaerobranca* species. In fact, the first microorganisms discovered to use Fe(III) as

1 electron acceptor during anaerobic growth were fermentative bacteria (Lovley 1991)
2 such as *Clostridium pasterurianum* and *Lactobacillus lactis*. Subsequent studies
3 found that a wide variety of fermentative microorganisms were capable of reducing
4 Fe(III) and other transition metals (Jones et al., 1984; Lehours et al., 2010). However,
5 it was suggested that iron reduction was only a minor pathway for electron flow for
6 fermentors (Lovley, 1991). Our results showed that strain CCSD-1 was capable of
7 growth when coupled with reduction of Fe(III) in smectite, however, further research
8 is necessary to truly understand the physiological role of Fe(III) reduction in
9 fermentative bacteria.

10 Compared with typical DIRB, under similar experimental conditions (e.g., 0.1
11 mM AQDS, 5 g/L N_{Au-2}, ambient temperature), the initial reduction rate (within the
12 first day) by CCSD-1 was much lower than that of N_{Au-2} by *S. putrefaciens* CN32
13 (0.029 mM/h vs. 0.08 mM/h) (Jaisi et al., 2005; Dong et al., 2009). Nevertheless, the
14 ultimate reduction extent (26.2%) was similar to that achieved by *S. putrefaciens*
15 CN32 (Jaisi et al., 2005; Zhang et al., 2007a). Incomplete reduction of structural
16 Fe(III) in various clay minerals has been observed for other types of bacteria (Kostka
17 et al., 1996; Ribeiro et al., 2009; Dong et al., 2009; Bishop et al., 2011; Dong, 2012;
18 Liu et al., 2011, 2012, 2014). The combined effects of electron transfer pathway,
19 elemental toxicity, and energetics might have been responsible for the incomplete
20 bioreduction of structural Fe(III) in clay minerals. In general, clay minerals carry a net
21 negative surface charge (Sposito et al., 1999) but some positive charge exists on clay
22 edges (Frissel and Bolt, 1962). Because of the overall negative charge of bacterial

1 surfaces, microorganisms probably preferably attach to clay edges due to electrostatic
2 attraction. Therefore, the electron transfer pathway should have been from the
3 smectite edge to the internal structure of nontronite (Dong et al., 2009; Ribeiro et al.,
4 2009; Stucki, 2011). In doing so, much of the structurally-coordinated Fe(III) in
5 nontronite may not be accessible to iron-reducing microorganisms. An electron shuttle
6 such as AQDS would facilitate electron transfer possibly via the interlayer of clay
7 minerals (Dong et al., 2009) and thus promote Fe bioreduction. However,
8 bioreduction of structural Fe(III) is still incomplete. In this case, elemental toxicity
9 may be an important factor. Due to partial reductive dissolution, cations (e.g., Fe^{2+}
10 and Al^{3+}) may be released into aqueous solution. Previous studies have demonstrated
11 that aqueous Fe^{2+} is toxic to cells and if sorbed to clay surfaces, Fe^{2+} could block the
12 electron transfer chain from microbes to mineral surfaces (Jaisi et al., 2007). These
13 mechanisms would further decrease the extent of Fe(III) reduction in clay minerals.
14 Furthermore, previous work revealed that Al^{3+} could be considered as another
15 inhibitor to iron reduction because of its toxicity (Wood, 1995).

16

17 ***4.2. Low-temperature feldspar formation***

18 Feldspar minerals, which are common constituents in most igneous and
19 high-grade metamorphic rocks, normally form at high temperature and high pressure
20 (Faure, 1992). As such, feldspars in sedimentary rocks are generally considered as
21 detrital minerals. However, by examining several hundred thin sections of
22 sedimentary rock samples, Kastner and Siever (1979) first noted that approximately 5

1 percent of all feldspars in sedimentary rocks had an authigenic origin, which were
2 formed at low temperature by reactions in aqueous solution. Unlike the detrital
3 feldspars with irregular shapes, the low-temperature feldspars have an euhedral
4 crystal shape in a sub-micrometer to micrometer size range (Kastner and Siever,
5 1979). Motivated by this suggestion, other studies have subsequently documented
6 minor but common occurrence of low-temperature feldspars in carbonate rocks and
7 sandstones (e.g., Hearn and Sutter, 1985; Molenaar and de Jong, 1987; Ramseyer et
8 al., 1993; Spötl et al., 1999; Rais et al., 2008). There have been two models proposed
9 to explain the low-temperature feldspar authigenesis: (a) isochemical transformation
10 (Kastner and Siever, 1979), and (b) the interaction between migrating brine and host
11 rocks (Hearn and Sutter, 1985; Spötl et al., 1999; Rais et al., 2008). In the isochemical
12 transformation model, authigenic feldspar forms through the burial transformation of
13 pre-existing detrital minerals in sediments. This model indicates that all the
14 constituents (Na, Ca, Al, Si, O, etc.) necessary for feldspar formation could be
15 supplied by the sediment itself (Kastner and Siever, 1979). For example, Al could be
16 released by the decomposition of montmorillonite, and Si might be supplied by clay
17 minerals or siliceous fossils. However, field evidence strongly argues against this
18 model. Specifically, it cannot well explain the restricted occurrence of
19 low-temperature feldspar in regional sedimentary rocks (Rais et al., 2008). If the
20 sediment itself serves as the essential donor of all constituents for feldspar
21 authigenesis, authigenic feldspar should be expected to be a common mineral in
22 sedimentary rocks. In contrast, the interaction model between migrating brines and

1 host rocks proposes that the cations (Na, K and Ca) in feldspar are derived from saline
2 fluids, and other constituents of feldspar are supplied from siliciclastic debris (e.g.,
3 clay minerals) within host rocks (Rais et al., 2008). However, an experimental study
4 by Small (1994) showed that the formation of feldspar through the reaction between
5 saline solution and smectite required high temperature and pressure (200 °C and 50
6 MPa). Therefore, there may be a natural catalyst to promote low-temperature feldspar
7 formation in sedimentary rocks.

8 Our present results demonstrated that plagioclase (Na-Ca feldspar) could be
9 synthesized at room temperature as a result of microbial reduction of structural Fe(III)
10 in smectite under alkaline condition. Given the fact that there is no feldspar detected
11 in either the original NAu-2 in the size range of 0.02-0.5 μm (Keeling et al., 2000;
12 Jaisi et al., 2005) or the abiotic control (Fig. 3A), the feldspar observed in our
13 experiments amended with strain CCSD-1 is therefore biogenic in origin. One
14 mechanism for the presence of biogenic feldspar could be that Al and Si released from
15 dissolved smectite combined with Na and Ca from the culture medium to form
16 feldspar under alkaline environment (pH=9.4). There may be two pathways in which
17 alkaliphilic iron reducers, e.g. strain CCSD-1, promoted feldspar formation: (a)
18 microbial reduction of Fe(III) in smectite may have led to partial dissolution of
19 smectite and the release of Al, Si and other structural cations into aqueous solution
20 (Dong et al., 2003; Liu et al., 2011), which re-combined to form feldspar under
21 alkaline condition; (b) The cell walls and EPS of alkaliphilic iron reducers may have
22 served as nucleation sites for feldspar formation and growth. It is well known that

1 dissolved Al and Si could preferentially sorb to bacterial cell walls and form Al-Si
2 complexes when a state of supersaturation is achieved (Konhauser and Urrutia, 1999).
3 Upon dehydration, the amorphous Al-Si complexes could be readily transformed into
4 poorly-ordered Al-silicates (Konhauser and Urrutia, 1999). It is reasonable to
5 speculate that Na and Ca from fluids (culture medium in our study) can be
6 incorporated into these Al-silicates, resulting in precursor feldspar phases at alkaline
7 pH. The EPS-feldspar association observed in our SEM images (Fig. 4A) highlights
8 the role of bacterial appendages in low-temperature feldspar formation.

9

10 **4.3. The S-I reaction**

11 As stated before, a variety of iron-reducing microorganisms including DIRB and
12 SRB, could catalyze the S-I reaction through bioreduction of Fe(III) in smectite.
13 Multiple lines of evidence from XRD, SEM and TEM observations presented here
14 also revealed the appearance of illite and mixed-layer smectite-illite in bioreduced
15 samples. A two-step mechanism is generally accepted to account for the biogenic illite
16 formation: reductive dissolution upon microbial activity and subsequent illite
17 precipitation and growth (Jaisi et al., 2011; Liu et al., 2014). Water chemistry (e.g.,
18 ionic strength, pH, and Al and K availability) and other factors (e.g., pressure and
19 temperature) probably play more important roles than the reduction extent and types
20 of microbes used (Jaisi et al., 2011). A previous study (Jaisi et al., 2011) studied
21 microbial reduction of Fe(III) in nontronite using mesophilic and thermophilic DIRB
22 and found that more illitic layers were formed under slightly higher pH conditions

(pH=8.4) even when the reduction extent was low. In the present study, the experimental conditions such as alkaline pH and sufficient K source were adequate to promote illite formation. In addition, the final reduction extent was also similar to that by typical DIRB. Therefore, it is not surprising to observe smectite illitization in bioreduced samples.

The family *Clostridiaceae* (*Firmicutes* group), to which strain CCSD-1 belongs, is widespread in the terrestrial deep subsurface (Zhang et al., 2005; Lin et al., 2006). Given the cosmopolitan status of smectite clay, undoubtedly, microorganisms can coexist and interact with smectite in subsurface sedimentary environments. Strain CCSD-1 from the deep subsurface tested here exhibited a similar or even stronger capability of iron reduction than that of surface or near-surface microorganisms (Roh and Moon, 2001; Roh et al., 2006). Microorganisms are therefore important agents in smectite illitization, especially at near ambient temperature. Such a microbially induced smectite illitization has been implicated either directly or indirectly in young and thermally immature marine sediments and mudstones (Masuda et al., 2001; Vorhies and Gaines, 2009; Dong et al., 2009).

4.4. Geological implications

As mentioned previously, several secondary minerals were observed in association with microbial respiration of iron-bearing smectite at near-neutral pH, including mixed-layered I-S, discrete illite, calcite, and silica (Fig. 6A). Among those secondary mineral products, thermodynamically metastable solid phases can undergo

1 transformation to more stable minerals as a consequence of burial diagenesis or
2 low-grade metamorphism. Hence, those minerals that display intergrown crystal
3 assemblages found in sedimentary rocks may be promising biomarkers for the
4 detection of neutrophilic iron-reducing microorganisms in geological times
5 (Sanz-Montero et al., 2009; Vorhies and Gaines, 2009). Different from neutrophilic
6 iron reducers (Dong et al., 2009), alkaliphilic iron-reducing microbes can convert
7 smectite to these minerals as well as feldspar (Fig. 6B). Biogenic feldspar formation
8 accompanied smectite illitization and other secondary mineral generation (e.g., silica
9 or its diagenetic product quartz) (Fig. 6B). Therefore, the mineral assemblage
10 consisting of low-temperature biogenic feldspar, illite, and quartz is proposed herein
11 as a biosignature for alkaliphilic iron reducers in the past. Such a model could be a
12 possible explanation for the occurrence of authigenic feldspar and illite in some
13 geological settings, such as Jurassic alkaline-saline Lake T'oo'dichi' (Turner and
14 Fishman, 1991; Potter-McIntyre et al., 2014).

15

16 5. Conclusions

17 A novel anaerobic alkaliphile isolated from the deep subsurface was capable of
18 reducing structural Fe(III) in nontronite, and the reduction extent was similar to that
19 of typical DIRB. XRD, Sybilla simulation, SEM and TEM evidence collectively
20 demonstrated that bioreduction triggered plagioclase (Na-Ca feldspar) formation and
21 smectite illitization. These results suggest that the mineral assemblage including
22 low-temperature feldspar and illite could be a potential biomarker for the activity of

alkaliphilic microorganisms in geological time.

Acknowledgments

This research is financially supported by grants from the National Basic Research Program of China (No. 2011CB808800), Natural National Science Foundation of China (Nos. 41030211 and 41302270), National Science Foundation (EAR 1148039), the 111 Project (No. B08030), a China Postdoctoral Science Foundation funded project (2013M531761) to Deng Liu, and the Fundamental Research Funds for the Central Universities (CUGL140815 and CUGL150409). The JEOL 2100 TEM used in this study was supported by NSF grant EAR-0722807. We are grateful to Editor-in-Chief and two anonymous reviewers whose comments improved the quality of the manuscript.

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Figure Captions:

Figure 1. (A) SEM image showing rod shaped CCSD-1 cells. White arrows indicate possible endospores; (B) Phylogenetic tree of CCSD-1 based on 16S rRNA gene analysis; (C) Effect of temperature on the specific growth of CCSD-1; (D) Effect of pH on the growth of CCSD-1.

Figure 2. Time course changes of total Fe(II) concentration (A) and cell concentration (B) in CCSD-1 inoculated systems with AQDS.

Figure 3. (A) XRD patterns for the ethylene glycolated abiotic control and bio-reduced samples showing interstratified I-S and plagioclase as the major secondary minerals (Sme, smectite; R2 I-S intermediate + discrete illite: R2 I-S + Ill; Pl, plagioclase); (B) Comparison of the experimental XRD pattern from bio-reduced sample (solid curves) and a calculated pattern (dash curves) based on Sybilla stimulation; (C) the Sybilla modeling results show the formation of R0, R2 I-S and illite after bio-reduction of smectite by strain CCSD-1.

Figure 4. SEM images and EDS compositions showing various changes of smectite as a result of bio-reduction by strain CCSD-1: residual smectite (grain a in A), plagioclase (grain b in A), illite (grain c in B) and silica (grain d in C). The arrows shown in figure A indicate CCSD-1 cells and their excreted EPS-like materials.

Figure 5. (A) TEM image of bioreduced smectite incubated with strain CCSD-1 showing a plagioclase particle confirmed with the inset SAED pattern. The bottom panel shows EDS composition of the biogenic plagioclase; (B) TEM image of bioreduced smectite showing illite packet with 10 Å and 12 Å nontronite lattice fringes. The inset EDS shows a typical illite composition of high Al/Si ratio and K content.

Figure 6. Schematic diagram illustrating secondary minerals associated with bioreduction of Fe(III) in smectite at near-neutral (A) and alkaline (B) pH. These biogenic precursors are predicted to form various mineral assemblages upon burial diagenesis and low-grade metamorphism which can be used as biosignatures for ancient microbial activity.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6