Title

Community structure of bacteria on different types of mineral particles in a sandy soil

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Abstract

Various types of mineral particles in a soil probably provide different microenvironments for microorganisms. The purpose of this study is to investigate whether different types of mineral in a soil harbor different bacterial populations. DNA was extracted from five types (quartz, feldspar, pyroxene, magnetite, iron-coated reddish brown particles) of sand-size mineral particles separated from a sandy soil, and was amplified for partial 16S rRNA gene by PCR. Twenty-nine to sixty-nine amplicons per each type of mineral were cloned and sequenced, followed by phylogenetic affiliation of the sequences. As a result, some types of bacteria were detected on all of the types of mineral including the orders *Rhizobiales*, *Bacillales*, and *Acidobacteriales*. In the case of *Acidobacteriales*, higher percentages were found on magnetite and quartz. Some taxa were restricted to specific types of mineral; the class *Actinobacteria* was found on pyroxene but not on quartz, and rarely on magnetite and feldspar. Bacterial diversity at the order level estimated by Chao1 value was higher in feldspar and pyroxene than the other three types of mineral. The UniFrac Significance test indicated that the differences in bacterial community structures among the particles were suggestive except that between feldspar and pyroxene. These results support the idea that different communities of bacteria

18 were associated with each of the mineral types.

19 **Keywords**

20 Bacterial community structure; Bacterial diversity; Microhabitat; Mineral particle; 16S
21 rRNA gene.

22

23 **INTRODUCTION**

24 A large number of different microorganisms live in soil. The number of species in
25 several grams of soil is estimated to be tens of thousands or more than one million (Gans
26 *et al.* 2005; Roesch *et al.* 2007). It is still unclear which factors are responsible for the
27 high microbial diversity in soil; however, like macroorganisms, high habitat
28 heterogeneity in a soil is assumed to play a role (Horner-Devine *et al.* 2004). For
29 example, in a paddy rice (*Oryza sativa* L.) field containing diverse habitats for
30 microorganisms, the respective habitats such as floodwater, soil, percolating water, and
31 rice straw have been shown to have distinct community structures of microorganisms
32 (Kimura and Asakawa 2006; Cahyani *et al.* 2007, 2008; Kikuchi *et al.* 2007).

33 A variety of mineral particles contained within a soil would be one of the sources
34 of microscale habitat heterogeneity for the resident soil microorganisms. Cahyani *et al.*

(2007, 2008) showed that specific bacteria inhabit iron mottles and manganese nodules formed in a rice field. Carson *et al.* (2009) revealed that three types of minerals which were buried in and taken from the soil after 70-day incubation harbored different bacterial communities estimated by ribosomal intergenic spacer analysis. Not in soil, but on rock surface, Gleeson *et al.* (2006) reported that the community structures of bacteria associated with different minerals were different. In their review, Uroz *et al.* (2009) concluded that the chemical composition of mineral particles affects the structure of the associated bacterial communities.

In our previous paper (Kotani-Tanoi *et al.* 2007), we revealed that the community structure of bacteria associated with sand-size particles in a soil is semi-specific to the type of their inhabiting mineral by comparing the PCR-DGGE profiles of 16S rRNA genes. In the present paper, more detailed community structures of the mineral-associated bacteria will be shown by phylogenetic affiliation of the sequences of their 16S rRNA genes with an aim of investigating whether different types of mineral in a soil harbor different bacterial populations.

MATERIALS AND METHODS

52 **Mineral particles**

53 Five types of sand-size particles with different appearances were isolated under a
54 stereomicroscope from the same soil sample as used in the previous study (Kotani-Tanoi
55 *et al.* 2007) (sand-dune Regosol, Entisol; pH (H₂O), 7.1; total carbon content, 6.8 g kg⁻¹;
56 total nitrogen content, 0.68 g kg⁻¹; soil texture, sand 96.7%, silt 3.3%, clay 0.0%). The
57 mineral species of the particles was estimated as quartz, magnetite, feldspar (orthoclase),
58 and pyroxene (enstatite and/or ferrosilite) based on their appearance under a
59 mineralogical microscope equipped with a polarizer and an analyzer (Olympus SZX12,
60 Tokyo). As the remaining one type was reddish brown and lost the color after treated
61 with HCl (pH 2.0) at 98°C for 15 min, it is possible that the reddish brown particles were
62 those coated with ferric oxide, regardless of the mineral species of particle inside.

63 **DNA extraction and sequencing of partial 16S rRNA genes**

64 Each particle was transferred separately to a microtube and washed four times with 0.2
65 mL of sterilized water using a vortex mixer for 10 s to remove microorganisms attached
66 loosely to the particle and extraneous matter on the surface of the particle. As we
67 considered that specific feature(s) in the structure of bacterial community of a certain
68 type of mineral appeared less evident if one particle was analyzed, several particles of the

69 same mineral type were combined for further analyses. Thus, DNA was extracted from
70 24 particles of quartz, 12 particles of magnetite, 10 particles of feldspar, 10 particles of
71 pyroxene, and 10 particles of the reddish brown particles, as well as from the whole soil
72 sample, by the same procedures described previously (Kotani-Tanoi *et al.* 2007). We
73 consider that no significant bacterial contamination occurred during the processes above;
74 no bacterial colony was detected after 70 days of incubation on 1/100 NB agar when
75 particles were picked up from the autoclaved soil sample, washed, and poured onto the
76 agar, while nearly a hundred or more colonies per particle were found when nonsterile
77 particles were examined.

78 PCR amplification for the bacterial 16S rRNA genes was achieved by the booster
79 PCR method (Ruano *et al.* 1989) under the same reaction conditions as described
80 previously (Kotani-Tanoi *et al.* 2007). The PCR primers used for the initial
81 amplification were 5'-AGAGTTTGATCCTGGCTCAG-3' (corresponding to positions
82 8-27 in *Escherichia coli* (Migula) Castellani and Chalmers numbering) and
83 5'-AAAGGAGGTGATCCAGCC-3' (corresponding to positions 1542-1525). For the
84 following amplification, the PCR primers 5'-AACGCGAAGAACCTTAC-3'
85 (corresponding to positions 952-968) and 5'-GGYTACCTTGTTACGACTT-3'

86 (corresponding to positions 1510-1492) were used. The amplicons of partial 16S rRNA
87 gene (positions 952-1510) were cloned and sequenced by the method described in Saito
88 *et al.* (2008).

89 **Analyses of the sequences**

90 Based on the partial 16S rRNA gene sequence (positions 969-1491), phylogenetic
91 affiliation of the clones at the order level and higher was performed by the naïve
92 Bayesian rRNA classifier program (Wang *et al.* 2007) provided by the Ribosomal
93 Database Project (Cole *et al.* 2009) (<http://rdp.cme.msu.edu/>). The taxonomic richness
94 of bacteria on each type of particles was estimated using Chao1 estimator, which was
95 calculated with EstimateS ver. 8.0 (Colwell 2006) based on the number of the order
96 detected. The UniFrac (Lozupone *et al.* 2006) was used to determine whether two
97 particle-associated bacterial communities differed significantly (Significance analysis)
98 and which bacterial group(s) had an excess of sequences in a particular type of particles
99 (Lineage-specific analysis). All the sequences were aligned with Clustal X 2.1 (Larkin
100 *et al.* 2007) to create a single phylogenetic tree for input into UniFrac.

101 **Accession numbers**

102 Sequences of partial 16S rRNA gene have been deposited in DDBJ/EMBL/GenBank with

103 accession numbers AB497599 to AB497978.

104

105 **RESULTS AND DISCUSSION**

106 A total of 380 amplicons of partial 16S rRNA gene, *i.e.*, 62 amplicons from quartz, 64
107 from magnetite, 29 from feldspar, 69 from pyroxene, 61 from reddish brown particles
108 and 95 from the whole soil sample, were cloned and sequenced. The results of the
109 sequence-based identification were shown as relative abundance of bacterial taxa on each
110 mineral (Table 1). The orders *Rhizobiales*, *Bacillales*, and *Acidobacteriales* were
111 detected from all six samples, but their relative abundance was different among the
112 samples. Clones assigned to *Verrucomicrobiales* were found in quartz and magnetite,
113 but not in feldspar, pyroxene, and reddish brown particles. Several clones from
114 pyroxene, feldspar, and magnetite were assigned to the class *Actinobacteria*, but none
115 from quartz and reddish brown particles. The UniFrac Significance test indicated that
116 except the pair of feldspar and pyroxene, any two types of particle differed significantly
117 in their bacterial communities ($P < 0.01$) although the differences were suggestive ($P <$
118 0.10) after correcting for multiple comparisons (Table 2). These results suggest that
119 bacterial taxa were unevenly distributed among the various types of mineral particles in

120 this soil.

121 The bacterial communities of quartz and magnetite were characterized by the
122 dominance of the phylum *Acidobacteria* (Table 1) and the low diversity at the order level
123 estimated by Chao1 (Table 3). An excess of *Acidobacteria* in magnetite was indicated
124 also by the UniFrac's Lineage-specific analysis (data not shown). Recent studies on the
125 bacterial communities based on the environmental DNA analyses indicate that the
126 phylum *Acidobacteria* is one of the most abundant bacterial groups in the environment
127 including soil (Janssen 2006; Ward *et al.* 2009). Indeed, in the present study,
128 acidobacterial sequences were recovered from all the types of mineral and the whole soil.
129 According to Janssen (2006), acidobacterial sequences make up 5 to 46%, with an
130 average of 20%, of the total sequences in libraries of 16S rRNA and 16S rRNA genes
131 prepared from soil bacterial communities. The high percentages of acidobacterial
132 sequences in the present study, *i.e.*, 35% for quartz's community and 42% for magnetite's,
133 both higher than the average reported by Janssen (2006), suggest that these minerals
134 provide such microsites that the phylum *Acidobacteria* favors. The abilities to adhere to
135 ferric iron-rich substrates (Johnson *et al.* 2008) and to catalyze iron redox reactions
136 (Ward *et al.* 2009) reported for acidobacterial strains may contribute to the dominance of

this phylum in the bacterial community of magnetite. Their ability to exhibit slow metabolic rates in nutrient-limited conditions (Ward *et al.* 2009) may be suitable for the survival on quartz. The bacterial community of quartz was also characterized by the presence of *Verrucomicrobia* and the absence of *Actinobacteria* although the reasons are unclear.

The bacterial community of reddish brown particles was characterized by the dominance of the orders *Burkholderiales* (31%) and *Bacillales* (28%). *Burkholderiales* was suggested to be enriched in reddish brown particles by the UniFrac's Lineage-specific analysis (data not shown). The expected presence of amorphous ferric oxides/oxyhydroxides coatings on the reddish brown particles attracts organic compounds (Violante *et al.* 2002; Wagai *et al.* 2009). Members of the order *Burkholdriales* have a variety of physiological traits and lifestyles (Vial *et al.* 2011); they are able to use a wide array of compounds as carbon sources (e.g., Chiarini *et al.* 2006; Morimoto *et al.* 2008; Saito *et al.* 2008) and are revealed to have efficient mineral weathering potentials (Calvaruso *et al.* 2010). These metabolic versatility may help them survive at the reddish brown particles where organic compounds accumulate.

In contrast to their dominance at reddish brown particles, no *Burkholderiales* clone

was found in the whole soil in this study. It seems to be in accordance with the previous report by Fulthorpe *et al.* (2008), in which *Burkholderia* was absent from the ten most abundant genera in their four soils analyzed by culture-independent, 16S rRNA gene techniques.

Bacillales clones were recovered from all the types of mineral and the whole soil sample in this study. According to Nicholson (2002), several *Bacillus* spp. have been isolated from the interiors of such locations as manganese rock varnish, desert rocks, deep subsurface boreholes, and near-surface granite formations. Flores *et al.* (1997) found *Bacillus* spp. on granite buildings and monuments that were slowly destroying. Together with these previous studies, the present result may suggest that some members of the order *Bacillales* are common residents on mineral surface.

As compared to the other three minerals, high values of Chao 1 were found for the bacterial community of pyroxene and feldspar (Table 3). Pyroxene and feldspar are known to be weathered more easily than quartz (Jackson and Sherman 1953), thus could supply more of bioavailable inorganic nutrients. Gleeson *et al.* (2006) suggested that mineral composition, especially, Al, Si, and Ca, has a significant impact on the structure of the bacterial communities associated with minerals of pegmatitic granites. The

present results may imply that such elements as become bioavailable more on pyroxene and feldspar than on quartz affect the degree of the diversity of the mineral-associated bacterial community in soil.

Several taxonomic groups such as *incertae sedis* TM7, the order *Caldilineales*, and the order *Legionellales*, appeared only in the whole soil, not in any of the five types of the particles examined. It is uncertain where in the soil the members of those taxa lived. As we picked up the sand mineral particles from the soil and washed them before extraction of DNA in this study, it is possible that they lived in the soil as free-living or associated with fine mineral particles or organic matter, or attached loosely rather than firmly to the sand particles. As members of these taxa have been often detected in biofilms and organic granules (Atlas 1999; Hugenholtz *et al.* 2001; Sekiguchi *et al.* 2003; Yamada and Sekiguchi 2009), the latter two might be more probable as sites for these groups.

In contrast, some taxa were not or rarely detected from the whole soil but detected from specific types of mineral. For instance, no *Planctomycetes* clone was found in the whole soil, whilst 3 out of 62 clones (5%) were found in quartz. Only 1 clone (1%) of *Verrucomicrobia* was found in the whole soil, whilst 4 clones (6%) found in quartz. It

suggests that investigation into specific type(s) of mineral is one of the good approaches to find rare or unknown microorganisms in soil.

In summary, the results in this study support the idea that different communities of bacteria are associated with each of the mineral types. It would be necessary for a more clear characterization of mineral-associated bacterial community to investigate the same types of minerals in other soil samples.

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Table 1 Relative abundance (%) of bacterial taxa of 16S rRNA gene fragment in those cloned from each type of mineral and whole soil

Phylum-Class-Order	Quartz (n = 62) [†]	Magnetite (n = 64)	Feldspar (n = 29)	Pyroxene (n = 69)	'Reddish' (n = 61)	Whole soil (n = 95)
Chloroflexi-Anaerolineae-Caldilineales						2
Chloroflexi-Chloroflexi-Chloroflexales		2	3			1
Nitrospira-Nitrospira-Nitrospirales		5				2
Proteobacteria-Alphaproteobacteria-Rhizobiales	2	16	10	6	5	12
Proteobacteria-Alphaproteobacteria-Rhodospirillales		3	3	6		1
Proteobacteria-Alphaproteobacteria-Sphingomonadales				1	2	
Proteobacteria-Alphaproteobacteria-Unknown	3			1		1
Proteobacteria-Betaproteobacteria-Burkholderiales	2		3	7	31	
Proteobacteria-Betaproteobacteria-Unknown	8	3	3	3	2	9
Proteobacteria-Gammaproteobacteria-Legionellales						1
Proteobacteria-Gammaproteobacteria-Pseudomonadales		2				1
Proteobacteria-Gammaproteobacteria-Xanthomonadales				3		
Proteobacteria-Gammaproteobacteria-Unknown		2				
Proteobacteria-Deltaproteobacteria-Myxococcales				1		1
Proteobacteria-Unknown-Unknown	6			1	3	2
Firmicutes-Bacilli-Bacillales	8	8	24	16	28	13
Firmicutes-Bacilli-Lactobacillales	2		3	1	5	
Firmicutes-Bacilli-Unknown			7	1	5	
Firmicutes-Clostridia-Clostridiales	6	2		1	2	5
Firmicutes-Unknown-Unknown	3		3	1	2	5
Actinobacteria-Actinobacteria-Actinomycetales			3	4		7
Actinobacteria-Actinobacteria-Rubrobacterales			3	3		1
Actinobacteria-Actinobacteria-Unknown		3		10		1
Planctomycetes-Planctomycetacia-Planctomycetales	5	2		1		
Acidobacteria-Acidobacteria-Acidobacteriales	35	42	10	20	7	6
Bacteroidetes-Sphingobacteria-Sphingobacteriales	2					
Verrucomicrobia-Verrucomicrobiae-Verrucomicrobiales	6	2				1
Gemmatimonadetes-Gemmatimonadetes-Gemmatimonadales				1		
(incertae sedis)-TM7						3
Unknown-Unknown-Unknown	11	11	21	7	10	23

[†]: number of clones

Table 2 *P*-value of UniFrac Significance test for each pair of particle-associated bacterial communities with the number of permutations set to 100. Values in normal letters are *P*-values corrected for multiple comparisons (upper right) and those in italics are raw *P*-values (lower left).

	Quartz	Magnetite	Feldspar	Pyroxene	‘Reddish’
Quartz	-	< 0.10	< 0.10	< 0.10	< 0.10
Magnetite	<i>0.00</i>	-	< 0.10	< 0.10	< 0.10
Feldspar	<i>0.00</i>	<i>0.00</i>	-	1.00	0.10
Pyroxene	<i>0.00</i>	<i>0.00</i>	<i>0.34</i>	-	< 0.1
‘Reddish’	<i>0.00</i>	<i>0.00</i>	<i>0.01</i>	<i>0.00</i>	-

Table 3 Estimated number (Chao1) of the orders in bacterial community of each type of mineral and whole soil

	Quartz	Magnetite	Feldspar	Pyroxene	'Reddish'	Whole soil
No. of sequences	62	64	29	69	61	95
No. of orders detected	14	14	13	21	12	21
Chao 1 value	16.0	17.8	27.0	32.3	15.0	30.0