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SHORT COMMUNICATION

Genera *Burkholderia* and *Lipomyces* are predominant aluminum-resistant microorganisms isolated from acidic forest soils using cycloheximide-amended growth media

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Abstract In acidic forest soils, microorganisms should adapt to toxicity of aluminum (Al), which is solubilized by acidity. We hypothesized that Al-resistant bacteria are diverse, especially in soils with high levels of Al, because these bacteria are expected to have adapted to Al stress over a long time. We isolated Al-resistant bacteria from acid forest soils using diluted tryptic soy broth agar plates with added Al and cycloheximide, and examined the relationship between their diversity and Al levels in soils. Based on 16S rDNA sequences, 10 out of 11 isolated bacteria were assigned to the genus *Burkholderia*, and 1 to the genus *Acinetobacter*. Although cycloheximide was added to the Al-enriched agar, yeasts were isolated from soils, and were examined. On the basis of ITS1, 5.8S rDNA, and ITS2 sequences, 13 out of 14 yeast isolates were assigned to *Lipomyces* sp. and 1 isolate to *Cryptococcus* sp. Diversity of Al-resistant bacteria was low in acidic forest soils, and was not related to Al levels in soils. Population numbers of Al-resistant microorganisms, however, increased with increasing Al levels.

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Introduction

Aluminum has a higher affinity for phosphorus (P) and slower reaction kinetics than calcium (Ca) or magnesium (Mg), and is thus a powerful inhibitor of many biological processes dependent on Ca and Mg (Macdonald and Martin 1988; Exley and Birchall 1992). Aluminum toxicity is a major problem in acid soils because Al solubility increases with decreasing soil pH (Sposito 1996). Acidic precipitation has therefore exaggerated the adverse effects of Al on plants and microorganisms in these soils. High levels of Al in soils may cause root damage and mineral imbalances in trees, and even result in forest decline (Godbold et al. 1988). Microorganisms are known to be more sensitive to Al toxicity than are trees (Joner et al. 2005). However, few studies have assessed the adverse effects of Al on soil microorganisms or their adaptation to Al stress (Piña and Cervantes 1996). In contrast, the environmental behavior of Al in soils (Sposito 1996), and the adaptation of plants to Al toxicity (Matsumoto 2000), have been well studied. Aluminum directly affects microorganisms through its fixation to cell walls, fixation to DNA via binding with P, and its toxic effects on enzymes (Robert 1995). It indirectly affects microorganisms through perturbation of Ca and Mg metabolism (Robert 1995), leading to reductions in microbial biomass, basal respiration, ATP levels, and protease activity in soils (Illmer et al. 2003). Al-resistant microorganisms are

therefore likely to play a key role in various microbially-mediated processes such as nutrient cycling in soils.

Bacterial Al resistance systems seem to be unspecific and passive (meaning that systems have another primary role, such as production of extracellular polysaccharides; Robert 1995). Bacteria have, however, developed sophisticated resistance mechanisms to various heavy metals, including efflux systems for cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn), and volatilization of mercury (Hg) (Silver and Phung 2005). No specific genes for Al resistance have so far been found in bacteria (Silver and Phung 2005). The most extensively investigated is *Pseudomonas fluorescens*, in which Al is secreted in association with oxalate and phosphatidylethanolamine by an energy-independent process (neither proton-motive force nor ATP hydrolysis), and is immobilized in the insoluble gelatinous precipitate (Hamel and Appanna 2003; Lemire et al. 2010).

Acidic forest soils with high exchangeable Al concentrations are widely distributed in Japan, and have developed through natural processes across long periods of time. We hypothesized that Al-resistant bacteria are diverse, especially in soils with high levels of Al, because these bacteria are expected to have adapted to Al stress in acidic forest soils over a long time. To examine this, we isolated and identified predominant Al-resistant bacteria from Japanese acidic forest soils.

Materials and methods

Soils

Soil samples were collected from A horizons in forests in Nagano Prefecture, Japan. Three soils were classified as allophanic Kuroboku soils (allophanic Andisols), and five soils were classified as Haplic Brown Forest soils (Inceptisols). Vegetation was predominately larch, *Larix kaempferi*, in five sites (A1, A3, B1, B2, and B3), Japanese red pine, *Pinus densiflora*, in one site (A2), Japanese chestnut, *Castanea crenata*, and Mongolian oak, *Quercus mongolica*, in one site (B4), and Veitch fir, *Abies veitchii*, and Maries fir, *Abies mariesii*, in one site (B5). Sampling sites were located at altitudes between 480 and 2,050 m. The mean annual precipitation at meteorological stations adjacent to sampling sites ranged from 941 to 2,152 mm, and the air temperature from 7.1 to 16.5°C.

Each soil sample was sieved through a 2-mm mesh and homogenized well. A portion of each sample was air-dried for chemical analysis, while the remainder was kept field-moist at 4°C. Soil properties were determined using standard methods as described elsewhere (Kunito et al. 2009, 2011); these are shown in Table 1. Exchangeable Al, a fraction toxic to microbes (Illmer et al. 1995) and plants (Saigusa et al. 1980), was extracted using 1 M KCl, and analyzed using an atomic absorption spectrometer 5100ZL (Perkin Elmer, Tokyo). All data are expressed on a dry weight basis.

Isolation and identification of Al-resistant microorganisms

Moist soil samples were dispersed in sterile tap water using a Waring blender 500 C (Sakuma, Tokyo), and the resulting slurry was decimally diluted with sterile tap water. Samples were spread on a 15-fold-diluted TSB agar plate (tryptic soy broth, 2 g; agar, 10 g; cycloheximide, 50 mg; distilled water, 1 L; pH 4.0), with added Al at 1.5 mM. The Al level in the medium selected about 10% of the microbial population growing on a 15-fold-diluted TSB agar plate (pH 4.0) without Al. For preparation of the diluted TSB agar plate, an acidified TSB medium with HCl, an $AlCl_3$ solution, and an

Table 1 Properties of forest soils used

Soil no.	Latitude (N)	Longitude (E)	Altitude (m)	Soil order (USDA)	Soil group (Japan)	pH	Organic C (%)	Total N (%)	Exchangeable Al (mg kg^{-1})
A1	36°24′	138°03′	1,510	Andisol	Allophanic Kuroboku soil	6.1	5.2	0.35	1.06
A2	35°55′	138°02′	880	Andisol	Allophanic Kuroboku soil	5.2	8.2	0.69	56.7
A3	35°50′	137°52′	1,300	Andisol	Allophanic Kuroboku soil	4.9	8.6	0.54	145
B1	36°31′	138°10′	480	Inceptisol	Haplic Brown Forest soil	6.9	2.9	0.23	0.45
B2	35°51′	137°50′	1,560	Inceptisol	Haplic Brown Forest soil	4.9	8.8	0.69	104
B3	35°50′	137°50′	1,870	Inceptisol	Haplic Brown Forest soil	4.4	10.2	0.69	146
B4	36°26′	138°20′	1,100	Inceptisol	Haplic Brown Forest soil	4.5	18.0	1.0	217
B5	35°50′	137°49′	2,050	Inceptisol	Haplic Brown Forest soil	4.1	21.9	1.1	436

agar solution were separately autoclaved to prevent hydrolysis of the agar (Kanazawa and Kunito 1996). After cooling to 50ºC, these three solutions were mixed, and filter-sterilized cycloheximide was added. The pH of the resulting agar plate was confirmed as 4.0 in a preliminary experiment. After 10 days incubation at 25°C, five colonies were randomly isolated from each soil sample, and subjected to single colony isolation. Single colony isolation, however, was only partially successful (Table 2) because of obstruction by fungal growth on the plate, despite the addition of cycloheximide.

Genomic DNA of isolated bacteria was extracted according to Tago et al. (2006), and the isolates were identified based on 16S rRNA gene sequences. These were amplified by PCR using universal primers; 27f (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′). PCR was performed in 10 μl of a mixture containing 1 μl of genomic DNA solution, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U of ExTaq polymerase (Takara Bioscience, Tokyo). Amplification was carried out over 30 cycles (denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min). Amplified fragments of 16S rDNA were purified using ExoSAP-IT (GE Healthcare Bioscience, Tokyo), and were sequenced directly. Although cycloheximide, an inhibitor of cytosolic protein biosynthesis in eukaryotes, was added to the Al-enriched agar, yeasts and not bacteria were isolated from four soils (Table 2). These isolates were identified based on ITS1, 5.8S rDNA, and ITS2 sequences. These were amplified using primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) after extraction of nuclear DNA by the method of Makimura et al. (1994). The PCR protocol and sequence analysis were as described above. Phylogenetic analysis was performed using CLUSTAL X version 2 (Larkin et al. 2007).

Table 2 Characteristics of aluminum-resistant bacteria and yeasts from forest soils

Isolates	Soil no.	Accession no.	Tentative identification	Doubling time (hr)		Ratio of doubling time (Diluted TSB+1 mM Al) / (Diluted TSB)
				Diluted TSB	Diluted TSB+1 mM Al	
Bacteria						
BA1a	A1	AB665296	*Acinetobacter* sp.	7.20	7.47	1.04
BA3a	A3	AB665286	*Burkholderia* sp.	3.31	4.19	1.27
BB4a	B4	AB665287	*Burkholderia* sp.	2.77	3.48	1.26
BB4b	B4	AB665288	*Burkholderia* sp.	2.86	3.12	1.09
BB4c	B4	AB665289	*Burkholderia* sp.	3.15	4.89	1.55
BB4d	B4	AB665290	*Burkholderia* sp.	3.30	5.56	1.68
BB4e	B4	AB665291	*Burkholderia* sp.	3.14	6.33	2.02
BB5a	B5	AB665292	*Burkholderia* sp.	3.35	3.51	1.05
BB5c	B5	AB665293	*Burkholderia* sp.	3.04	3.31	1.09
BB5d	B5	AB665294	*Burkholderia* sp.	2.73	3.35	1.23
BB5e	B5	AB665295	*Burkholderia* sp.	2.69	3.08	1.14
Yeasts						
YA2a	A2	AB665297	*Lipomyces* sp.	7.99	7.62	0.95
YA2b	A2	AB665310	*Cryptococcus* sp.	4.67	4.23	0.91
YA2c	A2	AB665298	*Lipomyces* sp.	6.42	7.14	1.11
YA2d	A2	AB665299	*Lipomyces* sp.	6.01	6.46	1.07
YA2e	A2	AB665300	*Lipomyces* sp.	5.33	5.45	1.02
YB1a	B1	AB665301	*Lipomyces* sp.	6.83	6.51	0.95
YB1b	B1	AB665302	*Lipomyces* sp.	6.92	7.49	1.08
YB2a	B2	AB665303	*Lipomyces* sp.	6.45	6.32	0.98
YB2b	B2	AB665304	*Lipomyces* sp.	6.03	6.19	1.03
YB2c	B2	AB665305	*Lipomyces* sp.	6.42	6.56	1.02
YB2d	B2	AB665306	*Lipomyces* sp.	6.20	5.83	0.94
YB2e	B2	AB665307	*Lipomyces* sp.	6.26	6.22	0.99
YB3a	B3	AB665308	*Lipomyces* sp.	6.17	6.99	1.13
YB3b	B3	AB665309	*Lipomyces* sp.	5.02	7.17	1.43

TSB, tryptic soy broth

Evaluation of Al resistance of isolates

Levels of Al resistance were evaluated using a ratio of doubling time in 1.5-fold-diluted TSB (pH 4.0) amended with Al to the doubling time of control cultures grown under the same conditions but in the absence of Al (Kunito et al. 2001). Isolates were incubated in the diluted TSB with 0 or 1 mM Al at 25ºC, and the absorbances of the cultures at 660 nm were read at specific intervals. It should be mentioned that in this evaluation, 1.5-fold-diluted TSB with 1 mM Al, instead of 15 fold diluted TSB with 1.5 mM Al used for isolation, was employed. This is because 15-fold-diluted TSB was used to isolate both oligotrophic and non-oligotrophic microorganisms, but all resultant isolates appeared not to be oligotroph, and because measuring a doubling time is easily conducted at 1.5-fold-diluted TSB with 1 mM Al due to a more rapid growth of the isolates in this condition.

Results

Properties of soils are shown in Table 1. Exchangeable Al showed a wide range of concentrations (0.45–436 mg kg^{-1}). The Al level significantly increased with decreasing soil pH ($y=3.27\times10^{7}\times0.0699^{x}$, $r^2=0.94$, $p<0.001$).

The numbers of colonies on diluted TSB agar plates with 1.5 mM Al added ranged from 3.9×10^4 to 4.6×10^6 per gram of dry soil, with a significant positive correlation between the number of Al-resistant colonies and the exchangeable Al concentration in the soils ($r^2=0.78$, $p<0.01$). In contrast, the number of colonies on a diluted TSB agar plate without Al (range, 3.4×10^5 to 1.5×10^8 per gram of dry soil) showed a weaker correlation with exchangeable Al concentration in the soil ($r^2=0.57$, $p<0.05$). Due to obstruction by fungal growth, only 25 isolates were able to be single colony purified from 8 soils (Table 2). In spite of the addition of cycloheximide, only 11 isolates were bacteria, and 14 isolates were found to be yeasts. Among the 11 bacterial isolates, the 16S rDNA sequences of 10 isolates showed high similarity with species in *Burkholderia*. The same sequences were found in strains BB4c and BB4d, BB5a and BB5c, and BB5d and BB5e (Fig. 1). The sequence similarities to *Burkholderia phenazinium* LMG 2247^{T} were 99.1% for strain BA3a, 99.0% for BB4b, 99.1% for BB4c and BB4d, 99.1% for BB4e, 99.2% for BB5a and BB5c, and 99.4% for BB5d and BB5e. Strain BB4a showed 98.5, 98.4, 98.0, and 97.8% sequence similarity to *B. phenazinium* LMG 2247^{T}, *B. terricola* LMG 20594^{T}, *B. sediminicola* HU2-65W^{T}, and *B. sartisoli* RP007^{T}, respectively. Our phylogenetic tree shows that strain BB4a was closely related to *B. sartisoli*,

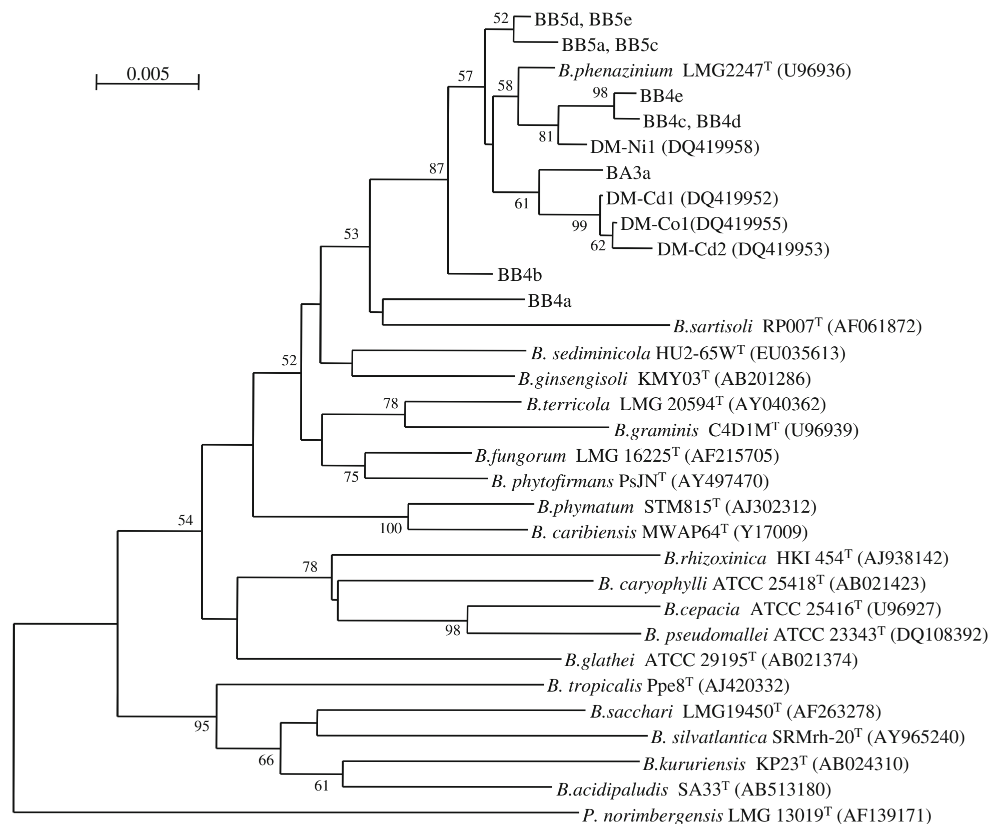

Fig. 1 Neighbor-joining phylogenetic tree constructed by using 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1,000 replicates) greater than 50% are shown at branch points. *Pandoraea norimbergensis* LMG 13019^{T} (AF139171) was used as an outgroup. *Bar* 0.005 substitutions per nucleotide position

while the other strains were most closely related to *B. phenazinium* (Fig. 1). Strain BA1a was assigned to the genus *Acinetobacter*. This strain was found to be most closely related to *A. guillouiae* DSM 590^T and *A. berezinae* ATCC 17924^T, with 16S rDNA sequence similarities of 99.4 and 98.9%, respectively. It clustered with these two species in our phylogenetic analysis (data not shown).

On the basis of ITS1, 5.8S rDNA, and ITS2 sequences, 13 out of 14 yeast isolates were assigned to *Lipomyces* sp. (Table 2). The 5.8S rDNA sequence was identical among all the *Lipomyces* isolates, and was also identical to the sequences of *L. tetrasporus* CBS 5910^T and *L. starkeyi* CBS 1807^T. The sequence of the ITS1 region in strains YA2a, YA2c, YA2d, YA2e, YB3a, and YB3b had group I introns; the conserved short sequences P, Q, R, and S (Cech 1988) were also found in this region (data not shown). Strain YA2b harbored an identical ITS1, 5.8S rDNA, and ITS2 sequence to that of *Cryptococcus podzolicus* strain CBS 6819^T, except that YA2b had one insertion in the ITS1 region.

In general, growth rates in diluted TSB were not significantly changed by addition of Al for the Al-resistant yeasts, but a longer doubling time was observed for the Al-resistant bacteria in diluted TSB with Al than for those without Al (Table 2). The ratio of doubling time in diluted TSB with 1 mM Al to that in diluted TSB without Al was significantly larger for the bacteria (mean±SD, 1.31±0.10) than for the yeasts (1.05±0.02) (Welch's t test, $p<0.05$), indicating a higher Al resistance in yeasts than bacteria. Although Al levels were low in soil samples A1 and B1 (Table 1), the resistance levels of the isolates were comparable with those of strains isolated from soils with higher Al levels (Table 2).

Discussion

In contrast with our expectation, diversity of Al-resistant bacteria was not related to Al concentrations in soils, and almost all bacteria found were assigned to the genus *Burkholderia* (Table 2). In addition, the Al resistance levels of isolates, evaluated using a change in doubling time by Al addition, were not associated with Al concentrations in soils.

According to Saigusa et al. (1980), Al-sensitive plants begin to become injured when the concentration of exchangeable Al exceeds 90 mg kg^{-1} dry soil. Five out of 8 of our soils exhibited higher Al levels than this threshold level (Table 1). It is also known that microorganisms are more sensitive to Al toxicity than are trees (Joner et al. 2005). However, populations of Al-resistant microorganisms increased with Al levels in the soils investigated. Al-resistant microorganisms are thus of potential importance as contributors to biological processes in acid soils with high Al levels.

The low diversity of Al-resistant bacteria, despite the long evolutionary time period of Al stress in these acid forest soils, might be due to the absence of specific genes for Al resistance in bacteria (Silver and Phung 2005). Bacteria may have difficulty developing a specific Al resistance system, because Al is present as insoluble forms at around pH 7 in cytoplasm, as pointed out by Fischer et al. (2002). Hence, unspecific and passive Al resistance might be inherited as a species-specific characteristic; resistant species, usually present in low frequencies in soils without Al toxicity, might become dominant under Al stress. In contrast, diversity of bacteria resistant to heavy metals is known to be very low in non-contaminated soils (e.g., Kunito et al. 1997a), but many soil bacteria adapt to heavy metal contamination by acquiring resistance through horizontal gene transfer, and diversity thus increases with time in contaminated soils (Kunito et al. 1998).

Another *Burkholderia* sp., *B. acidipaludis*, has also been isolated as an Al-resistant bacterium from acidic swamps (Aizawa et al. 2010). Several heavy metal-resistant bacteria within *Burkholderia* (e.g., DM-Cd1 and DM-Ni1) were very closely related to the Al-resistant bacteria that we isolated (Fig. 1). No published information was available for these heavy metal-resistant bacteria; only their 16S rDNA sequences were deposited in GenBank/EMBL/DDBJ databases. Meanwhile, several bacteria belonging to genera other than *Burkholderia* have been reported as Al-resistant: *Arthrobacter* sp. (Illmer and Mutschlechner 2004), *Flavobacterium* sp. (Konishi et al. 1994), *Pseudomonas fluorescens* (Lemire et al. 2010), and some within the genera *Acidocella*, *Acidiphilum*, and *Acidobacterium* (Wakao et al. 2002). It thus appears that the species of Al-resistant bacterium occurring in a particular place is dependent on the soil types and land use patterns that usually determine bacterial community composition in soils.

It should be mentioned that isolation of the Al-resistant *Lipomyces* sp., albeit with cycloheximide added to the medium, was owing to its resistance to both cycloheximide and acidity (Naganuma et al. 1999; Müller et al. 2007). In our previous studies, *Lipomyces* sp. were not isolated from soils using a medium with added cycloheximide and Cu or Zn (Kunito et al. 1997a, b, 2001). Because the pH of the medium was not acidic in our previous studies, it seems likely that the medium may need to be acidified for the isolation of *Lipomyces* sp. Alternatively, *Lipomyces* sp. may exhibit Al resistance, but be Cu and Zn sensitive. Similarly, a *Cryptococcus* sp. was isolated from acidic tea field soil as an Al-resistant yeast, using an acidic medium with added cycloheximide (Kanazawa et al. 2005). Hence, some species and/or strains of *Cryptococcus* may have resistance to both cycloheximide and acidity, and also to Al, in a similar manner to *Lipomyces* sp.

Our study has revealed that populations of Al-resistant microorganisms increased with increasing Al levels in acidic forest soils, although Al levels were greater than the threshold of toxicity to plants. Hence, such Al-resistant microorganisms may play a significant role in the biological processes within acid soils with high Al levels. The low diversity of resistant microorganisms might, however, cause their low functional diversity. Further studies are needed to evaluate their possible roles in nutrient cycling and ecosystem function in acidic forest soils with high Al levels.

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