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Identification and characterization of acidity-tolerant and aluminum-resistant bacterium isolated from tea soil

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An acidity-tolerant, aluminum resistant bacterium was isolated from tea soils in Kagoshima Experimental Station (Japan). Based on the morphological, physiological and biochemical characteristics and 16S rDNA nucleotide sequence analysis, the bacterium was identified as *Bacillus* sp. An 3 (DQ234657) in *Bacillus cereus* group. The bacterium was able to grow on S-LB plates (pH 3.7) with 1.0 g/L Al and survived in LB broth even at 10 g/L Al (pH 2.0). While cultured, the growth of the bacterial strain in LB liquid medium containing increasing concentrations of Al (0, 100 and 200 ppm), was inhibited by the presence of Al, especially at concentration of 200 ppm. The pH of culture medium without Al increased steeply and reached pH 7.0 after 10 days, meanwhile it was almost constant in the other cases. The elimination of Al from culture medium by the bacterium was also affirmed and it was more conspicuous at 100 ppm Al. Due to their tolerance to high acidity, resistance to and removal of a substantial amount of Al, the bacterium might be applicable in restoring acidic soils, particularly acidified tea garden soils.

Key words: Tea garden soil, acidity-tolerant bacterium, aluminum-resistant bacterium.

INTRODUCTION

Green tea (*Camellia sinensis*) is a nitrophilic crop. As a farming practice, large amounts of nitrogenous fertilizers, especially ammonium sulfate fertilizer, have been applied to tea soils in order to increase the amino acid content of tea leaves and produce an attractively colored, tasty tea. When tea plants absorb a large amount of ammonium, sulfate accumulates in the soil. Also, ammonium applied to tea soil is rapidly converted to nitrate by acid-tolerant autotrophic nitrifiers (Hayatsu and Kosuge, 1993). Consequently, a considerable quantity of nitrate and sulfate has gradually accumulated in soil (Nioh et al., 1993), decreasing the pH to 4.0 or even lower and raising remarkably soluble aluminum (Al) levels (Wang et al., 2010). In these conditions, the activity of soil micro-organisms decreases and tea plants are considered to accumulate a high level of Al, thus posing a serious threat to the health of consumers (Fung and Wong, 2004). The utilization of soil microorganisms, which are indis-pensable participants in biogeochemical cycles, should be considered as a potential solution. Following this trend, it is necessary to know what happens to microorganisms living in the extreme environment of the tea garden

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soil or how microorganisms cope with the unfavorable conditions of this extreme environment. In other words, the study of the microbial ecology of the extremely acidic tea soil has promised to provide insights into environmental and applied aspects of indigenous microorganisms.

However, there have been few studies related to this aspect which are nitrification and autotrophic nitrifying bacteria in acid tea soils (Walker and Wickramasinghe, 1979; Hayatsu and Kosuge, 1993), acidity- tolerant and Al- resistant microorganisms (Konishi et al., 1994; Nioh et al., 1995; Kanazawa and Kunito, 1996; Kawai et al., 2000; Kanazawa et al., 2005; Takashi et al., 2012; Wang et al. 2013; Chao et al., 2013) and microbial activities in tea soil (Nioh et al., 1993; Hayatsu, 1993; Kamimura and Hayano, 2000; Koga et al., 2003; Han et al., 2007).

Therefore, this study purports to fill the gap of the microbial ecology of the extremely acidic tea soil. It aims at identifying and characterizing acidity-tolerant and Al-resistant bacteria isolated from acidic tea garden soil in Kagoshima (Japan).

MATERIALS AND METHODS

Soil samples

Samples of Kuroboku (high-humic Andosol), Akahoya (light-colored Andosol), Kuroniga (thick high-humic Andosol), Andesite-derived (weathered soil of Neogene layer in Tertiary Period) and Sedimentary rock-derived (weathered soil of Shimantogawa layer in Jurassic Period) soils were collected from tea gardens at a depth of 0 to 20 cm at the Kagoshima Tea Experimental Station. All the fresh soil samples were passed through a 2 mm mesh sieve (JIS standard), dried for 24 h, passed through a 0.5 mm mesh sieve (JIS standard) and kept in closed glass bottles for storage at 5°C.

Soil properties

Moisture content was based on the gravimetric loss of free water associated with heating to 105°C for 24 h. The pH (H_20) was measured with a PB-20 Sartorius Basic pH Meter and expressed as the ratio of air-dried soil to solution: 1:2.5. The amounts of total C and total N were determined using a N-C analyzer. The water-soluble Al in soils was extracted with pure water (1:20), followed by shaking for 2 h (Iwasaki et al., 1993), diluted with 1% nitric acid and then quantified by using Inductively coupled plasma-mass spectrometry (ICP-MS).

The total number of microorganisms was estimated by the dilution agar plate method on NA medium (beef extract 5 g, peptone 10 g, NaCl 5 g, agar 15 g, water up to 1000 ml, pH 4.0 and 7.0), for three to five days at 30°C. The coarse organic substances in the soil samples were dissociated by dispersion using a Waring Blender at 16,000 rpm for 3 min (Kanazawa et al., 1986). Cycloheximide was also spread on the surface of the plates to prevent the overgrowth of several rapidly growing fungi, which would restrict the growth of slower-growing molds.

Isolation of acidity-tolerant and Al-resistant bacteria

Bacteria tolerant to acidity and resistance to Al was isolated as follows. Autoclaved non-acidic field soil (100 g) was added to 1 L of distilled water and agitated for 30 min at 100 rpm. The mixture was filtered using a 0.50 µm filter. The filtrate was mixed with LB liquid medium (0.05% peptone, 0.025% yeast extract and 1% NaCl) to produce S-LB liquid medium. After this medium was autoclaved at 121°C for 15 min, Al sterilized using a 0.20 µm filter was added to a final concentration of 100 ppm, and the medium was adjusted to pH 3.7. The acidic tea soils (1 g) were added to 10 ml of this, and the culture was performed on a shaker at 150 rpm, 30°C for 7 days. The resulting bacterial strains were purified by incubation on S-LB agar plates (pH 7.0) (Konishi et al., 1994) (Method I). On the other hand, bacterial strains were directly isolated by the dilution agar plate method on S-LB (Method II) or LB agar plates (Method III), containing Al at a concentration of 100 ppm (pH 3.7). All the above-isolated bacterial strains were then transferred to S-LB agar plates containing Al concentrations of 200 to 1000 ppm (pH 3.7) and incubated at 30°C for seven days in order to screen for resistance to Al. Although small amount of yeast extract (0.2 g/L) and peptone (0.5 g/L) were included in the S-LB agar plates, their effects on the existence of inorganic monomeric Al were negligible (Kawai et al., 2000). The bacterium with higher ability to resist Al was selected for further analysis.

Identification

Identification was based on a morphological, physiological and biochemical characterization and phylogenetic analysis.

Morphological, physiological and biochemical characteristics

The tests were investigated on cultures grown at 30°C for 48 h. The bacterium was examined with an optical microscope for its cell form and size, Gram reaction, spore formation and motility. Colony form was observed on a medium plate. The catalase reaction, oxidase reaction, acid or gas production from glucose and oxidation or fermentation (O/F) of glucose were tested (Barrow and Feltham, 1993). Besides, physiological and biochemical characteristics were also determined using API 50 CHB kit (bioMerieux, Lyon, France) consisting of 49 carbohydrates of API 50 CH strip associated with the API 20 E strip.

Phylogenetic analysis

Colonies which developed on LB agar plates after 48 h at 30°C were harvested for analysis. InstaGene matrix was used for extraction and purification of genomic DNA, following its protocol. The nucleotide sequence (1500 to 1600 bp) of 16S rDNA of the isolate was amplified by PCR. The extracted genomic DNA acted as a template. Primers 9F and 1510R were added to Ready-To-Go PCR beads (Amersham Pharmacia Biotech, NJ, USA) which consist of deoxynucleotides, Taq DNA polymerase and PCR buffer to produce a complete PCR mixture. The nucleotide sequence of the amplified 16S rDNA was determined with an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit. This kit was used with a GeneAmp PCR Systems 9600 thermal cycler and ABI Prism 3100 DNA Sequencer (Applied Biosystems, CA, USA). Eight kinds of sequence primers were used for the cycle sequencing. The sequences were screened for repeats, using an Auto Assembler 2.1 (Applied Biosystems, CA, USA) to rule out overlaps. The nucleotide sequence was analyzed by using MicroSeq Microbial Identification System Software V.1.4.1 (Applied Biosystems, CA, USA). MicroSeq Bacterial Full Gene Library v.0001 (Applied Biosystems, CA, USA) acted as a sequence database in similarity searches using the BLAST system (Saitou and Nei, 1987). Subsequently, a Neighbor-Joining molecular phylogenetic tree was constructed (Altschul et al., 1997). Then, in order to acquire more information, a similarity

search with the international nucleotide sequence database offered by U.S. National Center for Biotechnology Information (NCBI) using the BLAST was carried out. The nucleotide sequence data was submitted to GeneBank/DDBJ/EMBL for the accession number.

Bacterial tolerance to acidity and Al

To minimize the possible effect of soil eluate on the initial Al concentrations in the culture medium, the LB liquid medium was used in the following studies. $Al_2(SO_4)_3$ solution filtered with a sterilized filter (0.20 µm pore size) was added to the LB liquid medium autoclaved at 121°C for 15 min to final concentrations of 0.1 to 50 g/L^1, and the pH of the medium was adjusted to 2.0, 2.5, 3.0 and 3.5. The bacterial suspension (1 ml) was then inoculated into the medium and cultured by shaking at 150 rpm, 30°C for 7 days. After that, 50 µL of each culture was transferred onto LB plates in the absence of Al and heavy metals (pH 7.0), and continuously cultured at 30°C for three days. A positive test result under given culture conditions was affirmed via the development of colonies on these plates after incubating.

Culture conditions, bacterial growth and changes in medium pH

Cultures of 100 ml LB medium containing various concentrations of Al (pH 3.5) were inoculated with 1 ml of bacterial suspension and incubated by shaking at 150 rpm, 30°C for 10 days. During bacterial growth, the change of medium pH was measured with a PB-20 Sartorius Basic pH meter and the number of bacterial cells was counted by the dilution method on LB agar plates (pH 7.0).

Quantification of Al eliminated from culture medium

The spent culture supernatant was separated by centrifugation at 12,000 rpm for 10 min, then filtered with a sterilized filter (0.20 µm pore size), diluted with 1% nitric acid and subjected to ICP-MS analysis to determine the amount of Al remaining in the spent culture medium.

All the values represented the means of three independent experiments and were plotted along with their respective standard deviations. Differences of means were tested with Turkey-Kramer's method.

RESULTS

Soil properties

Several soil properties were determined (Table 1). The pH of tea soils varied in the range of 2.69-4.18. Soluble Al levels were significantly higher in the Kuroboku and Akahoya soil samples than in the other samples. The numbers of acidity tolerant microorganisms in the Kuroboku and Kuroniga samples probably increased due to the high acidity.

Isolation of acidity-tolerant and Al-resistant bacteria

Based on the differences in colony form, 41 bacterial strains which were able to tolerate pH 3.7 and 100 ppm Al was initially isolated. The result of the subsequent screening shows that two of these strains, namely Kb 1 and An 3, were able to grow on S-LB plates in the presence of 1000 ppm Al (Table 2). Therefore, strain An 3 derived from the Andesite-derived soil sample (pH 4.18) was selected for further research.

Identification

Morphological, physiological and biochemical characteristics

A photograph of strain An 3 is shown in Figure 1. The strain was a Gram-positive rod, 1.0 x 2.0-3.0 µm in cell size. This strain had motility and spore formation and was positive for both catalase and oxidase reactions. The characteristics of strain An 3 presented in Table 3 seemed to be in agreement with those of *Bacillus* genus. However, it was unlikely that this strain belonged to *B. mycoides* or *B. anthracis* which is included in the *B. cereus* group because they have no motility (Barrow and Feltham, 1993; Sneath et al., 1984). This is different from the above-mentioned suggestion based on the result of the nucleotide sequence analysis.

Besides, in physiological and chemical tests using the API 50 CHB kit, fermentation by strain An 3 of carbohydrate substrates such as ribose, glucose, fructose, arabinose, salicin, cellobiose, etc was detected, whereas that of others such as xylose, galactose, mannose, melibiose, raffinose, etc was not detected (Table 4). These characteristics of strain An 3 appeared to be similar to those of *B. cereus* and *B. thuringiensis* which were also contained in the *B. cereus* group. Although strain An 3 was considered to be closely related to *B. cereus* based on positivity for urease activity, however, their negativity of acetoin reaction (VP) was different.

In addition, in supplementary tests, strain An 3 was found to be positive in hemolysis, lecithinase and anaerobiosis, and negative in crystalline inclusion (Table 5). Based on these results, the possibility that strain An 3 belongs to *B. cereus* was greatest.

16S rDNA nucleotide sequence analysis

The nucleotide sequence of 16S rDNA of the bacterium was determined and presented in Figure 2. The result of the homology search with the MicroSeq Bacterial Full Gene Library using the BLAST system showed that the 16S rDNA base sequence of strain An 3 had more than 99% homology with that of *B. thuringiensis, B. cereus* and *B. mycoides* (Table 6). The result with the International Nucleotide Sequence Database using BLAST indicated 99.8% homology in 16S rDNA sequence with *B. cereus* H1439. Moreover, the first 20 hits in this

Table 1. Some properties of tea garden soil samples.

Soil sample	Depth (cm)	Moisture (%)	pH (H_2O)	Total C (g kg^{-1})	Total N (g kg^{-1})	C/N	Water soluble Al (mM kg^{-1})	Total number of microorganisms (10^6 g^{-1}) on NA medium	
								pH 4.0	pH 7.0
Kuroboku	0-20	28.32	2.69	9.57	0.582	16.4	9.45 ± 0.39	0.16	28.67
Kuroniga	0-20	26.91	3.11	17.36	0.880	19.7	6.77 ± 0.12	0.23	29.46
Akahoya	0-20	34.68	3.93	5.39	0.456	11.8	9.51 ± 0.35	0.12	31.38
Andesite-derived soil	0-20	38.48	4.18	2.70	0.270	10.0	2.77 ± 0.16	0.13	19.51
Sedimentary rock-derived soil	0-20	62.38	4.07	3.48	0.348	12.2	1.03 ± 0.40	0.09	3.90

homology list were related to *B. cereus* and *B. thuringiensis* (Table 7). Therefore, the possibility that strain An 3 belongs to *B. thuringiensis, B. cereus* or *B. mycoides* may be considered. However, since 16S rDNA nucleotide sequences of strain An 3 and these species do not entirely match, that the strain is closely related to another systematically different strain could not be absolutely excluded.

In general, five species, *B. thuringiensis*, *B. cereus*, *B. mycoides, Bacillus weihenstephanensis* and *B. anthracis* (Skerman et al., 1980) (anthrax, Bio Safety Level 3), are assigned to the *B. cereus* group with close relationships. In the Neighbor-joining phylogenetic tree constructed using MicroSeq (Figure 3), the cluster formed by strain An 3, *B. thuringiensis*, *B. cereus* and *B. mycoides* was considered to be the cluster of the *B. cereus* group (*B. weihenstephanensis* and *B. anthracis* were not registered in MicroSeq).

The 16S rDNA nucleotide sequence of strain An 3 has been deposited in the DDBJ/EMBL/GenBank database with the accession number DQ234657.

To sum up, the isolate may be identified as *Bacillus* sp. An 3 (with accession number DQ234657), part of the *B. cereus* group and related to *B. cereus, B. weihenstephanensis* or *B. thuringiensis*.

Tolerance to acidity and resistance to Al

Bacterial acidity tolerance and aluminum resistance in LB liquid medium were investigated. *Bacillus* sp. An 3 could survive in the presence of Al and low pH. As shown in Table 8, it could survive in the presence of Al up to 10 g/L at pH 2.0. This suggested that the strain was markedly tolerant to high acidity and resistant to Al.

Bacterial response to increasing concentrations of Al in culture medium

In this study, the growth of *Bacillus* sp. An 3 was influenced by the presence of Al in the culture medium, especially at an initial concentration of 200 ppm (Figure 5). In addition, during the growth, the pH of the culture medium without Al increased steeply and reached about 7.0 after 10 days, meanwhile it was almost constant at Al concentrations of 100 and 200 ppm (Figure 4). The result of the investigation on microbial elimination of Al from the culture medium showed that Al was removed by *Bacillus* sp. An 3 and it was more significantly conspicuous in the presence of 100 ppm Al than in the presence of 100 ppm Al (Figure 6).

DISCUSSION

Aluminum comprises 8.3% of the earth crust and is the most abundant metal and the third most abundant element after oxygen (45.5%) and silicon (25.7%). Aluminum appears in the Al^{3+} oxidation state and aluminum minerals are almost insoluble at neutral pH. As the pH drops below 5.5, however, Al-containing materials begin to dissolve. High levels of soluble Al in soils become toxic to plants and microorganisms (Mossor-Pietraszewska, 2001; Slattery et al., 2001). In order to deal with this, some microorganisms have developed mechanisms to tolerate high acidity and resistance to Al-stress conditions. In fact, a number of microorganisms tolerant to high acidity and resistant to Al from acidic soils have been isolated and identified (Konishi et al., 1994; Kanazawa and Kunito, 1996; Kawai et al., 2000; Nguyen et al., 2001; Kanazawa et al., 2005). However, it is remarkable that most of the microorganisms isolated were fungi and yeasts. This may be ascribed to the fact that fungi and

Table 2. Resistance to Al of acidity-tolerant bacteria.

Soil	Strain	Method of isolation	Al concentration (ppm)					
			100	200	300	400	500	1000
Kuroniga	Kn 1	(I)	+	-	-	-	-	-
	Kn 2	(II)	+	+	-	-	-	-
	Kn 3		+	+	-	-	-	-
	Kn 4		+	+	+	-	-	-
	Kn 6	(III)	+	+	+	-	-	-
	Kn 7		+	+	+	-	-	-
	Kn 8		+	+	+	+	-	-
	Kn 9		+	+	+	+	-	-
Kuroboku	Kn 10	(I)	+	+	-	-	-	-
	Kb 1		+	+	+	+	+	+
	Kb 2		+	+	+	+	+	-
	Kb 3	(II)	+	+	+	+	-	-
	Kb 4		+	+	-	-	-	-
	Kb 5		+	+	+	+	-	-
	Kb 6	(III)	+	+	+	+	+	-
	Kb 7		+	-	-	-	-	-
	Kb 8		+	+	+	+	+	-
Akahoya	Ah 1	(I)	+	+	-	-	-	-
	Ah 2		+	-	-	-	-	-
	Ah 3	(II)	+	+	-	-	-	-
	Ah 4	(III)	+	-	-	-	-	-
	Ah 5		+	+	+	-	-	-
	Ah 6		+	-	-	-	-	-
Andesite-derived soil	An 1	(I)	+	+	+	-	-	-
	An 2		+	+	+	+	+	-
	An 3		+	+	+	+	+	+
	An 4	(II)	+	+	+	-	-	-
	An 5		+	+	+	+	+	-
	An 6		+	+	+	+	-	-
	An 7	(III)	+	+	+	+	-	-
	An 8		+	+	+	+	-	-
	An 9		+	+	+	+	+	-
	An 10		+	+	+	+	-	-
	An 11		+	+	+	-	-	-
	An 12		+	+	+	+	+	-
Sedimentary rock-derived soil	Ts 1	(I)	+	+	+	+	-	-
	Ts 2	(II)	+	+	-	-	-	-
	Ts 3	(III)	+	+	+	+	-	-
	Ts 4		+	+	+	+	+	-
	Ts 5		+	+	+	+	-	-
Total	41		41	35	29	22	12	2

+ , Colonies; -, no colony grown at respective culture conditions.

yeasts are generally more tolerant to acidity than bacteria (Myrold and Nason, 1992; Pina and Cervantes, 1996). In addition to the acid- and Al- tolerant bacterial strain which was isolated and identified as *Flavobacterium* sp.

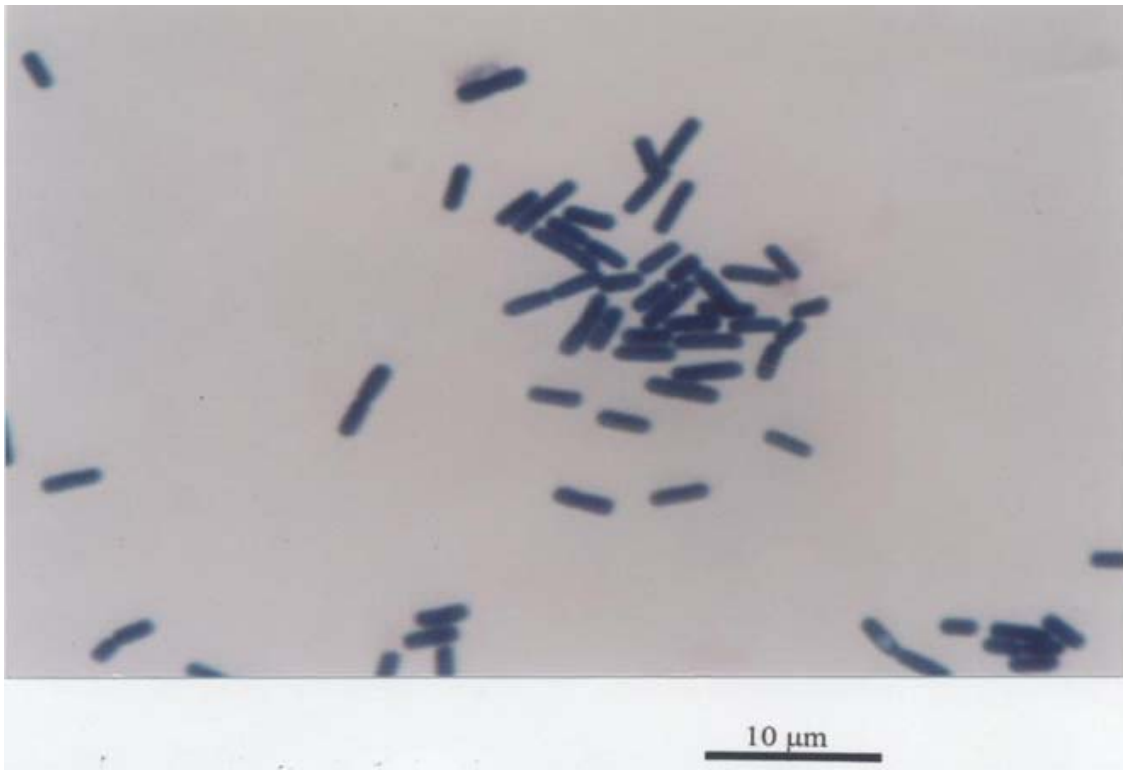

Figure 1. Microscopic characteristics of strain An 3.

Table 3. Morphological, physiological and biochemical tests.

Content		Strain An 3
Culture temperature (°C)		30
C Cell form and size (µm)		rod (1.0 x 2.0-3.0)
Gram reaction		+
Spore formation		+
Gliding motility		+
Colonial morphology		Culture medium: LB agar Culture time: 48 h Diameter: 1.0-2.0 mm Color: cream Form: ellipse Elevation: convex Margin: undulate Surface: smooth Opacity: opaque Texture: butter-like texture
Growth at (°C)	37	+
	45	-
Catalase production		+
Oxidase production		+
Acid/gas production from glucose		- / -
O/F test (Oxidation/Fermentation)		- / -

+, Positive; -, negative.

Table 4. Physiological and biochemical tests using API 50CHB Kit for the An 3 strain.

Tube	Test	Active ingredients	Reaction tested	Results
0		Control		−
1	GLY	GLYcerol	F/O	+
2	ERY	ERYthritol	F/O	−
3	DARA	D-ARAbinose	F/O	−
4	LARA	L-ARAbinose	F/O	−
5	RIB	D-RIBose	F/O	+
6	DXYL	D-XYLose	F/O	−
7	LXYL	L-XYLose	F/O	−
8	ADO	D-ADOnitol	F/O	−
9	MDX	Methyl-ßD-Xylopyranoside	F/O	−
10	GAL	D-GALactose	F/O	−
11	GLU	D-GLUcose	F/O	+
12	FRU	D-FRUctose	F/O	+
13	MNE	D-MaNnosE	F/O	−
14	SBE	L-SorBosE	F/O	−
15	RHA	L-RHAmnose	F/O	−
16	DUL	DULcitol	F/O	−
17	INO	INOsitol	F/O	−
18	MAN	D-MANnitol	F/O	−
19	SOR	D-SORbitol	F/O	−
20	MDM	Methyl-αD-Mannopyranoside	F/O	−
21	MDG	Methyl-αD-Glucopyranoside	F/O	−
22	NAG	N-AcetylGlucosamine	F/O	−
23	AMY	AMYgdalin	F/O	+
24	ARB	ARButin	F/O	−
25	ESC	ESCulin ferric citrate	F/O	+
26	SAL	SALicin	F/O	+
27	CEL	D-CELlobiose	F/O	+
28	MAL	D-MALtose	F/O	+
29	LAC	D-LACtose (bovine origin)	F/O	−
30	MEL	D-MELibiose	F/O	−
31	SAC	D-SACcharose (sucrose)	F/O	−
32	TRE	D-TREhalose	F/O	+
33	INU	INUlin	F/O	−

Table 5. Supplementary tests for bacterial identification.

Content	Strain An 3
Hemolysis test	+
Lecithinase activity	+
Crystalline inclusion	−
Anaerobiosis	+

(Konishi et al., 1994), in the present study, *Bacillus* sp. An 3 was able to survive in LB liquid medium containing a concentration of 10 g L^{-1} Al at pH 2.0. However, because of the various culture media, incubation conditions and assessment methods employed, it is difficult to make comparisons of Al resistance among bacteria from different studies.

It was reported that the bacterial adaptation to changes of medium pH may refer to the synthesis of an array of new proteins as part of what has been called their acidic tolerance response (Lansing et al., 2001). Furthermore, it was also proposed that either a high internal buffering capacity or reduced membrane permeability might play a role in pH homeostasis (Ian, 1985).

When pH decreases to 5.0 or lower, Al becomes soluble and toxic to microorganisms. The toxic effect of Al may be due to the substitution of essential metal ions at critical sites in the cell (Ganrot, 1986). However, the molecular mechanism of the toxicity has not been clarified. Here, the growth of *Bacillus* sp. An 3 was

1	gagtttgatc	ctggctcagg	atgaacgctg	gcggcgtgcc	taatacatgc
51	aagtcgagcg	aatggattra	gagcttgctc	tyawgaagtt	agcggcggac
101	gggtgagtaa	cacgtgggta	acctgcccat	aagactggga	taactccggg
151	aaaccggggc	taataccgga	taayattttg	aactgcatgg	ttcgaaattg
201	aaaggcggct	tcggctgtca	cttatggatg	gacccgcgtc	gcattagcta
251	gttggtgagg	taacggctca	ccaaggcaac	gatgcatagc	cgacctgaga
301	gggtgatcgg	ccacactggg	actgagacac	ggcccagact	cctacgggag
351	gcagcagtag	ggaatcttcc	gcaatggacg	aaagtctgac	ggagcaacgc
401	cgcgtgagtg	atgaaggctt	tcgggtcgta	aaactctgtt	gttagggaag
451	aacaagtgct	agttgaataa	gctggcacct	tgacggtacc	taaccagaaa
501	gccacggcta	actacgtgcc	agcagccgcg	gtaatacgta	ggtggcaagc
551	gttatccgga	attattgggc	gtaaagcgcg	cgcaggtggt	ttcttaagtc
601	tgatgtgaaa	gcccacggct	caaccgtgga	gggtcattgg	aaactgggag
651	acttgagtgc	agaagaggaa	agtggaattc	catgtgtagc	ggtgaaatgc
701	gtagagatat	ggaggaacac	cagtggcgaa	ggcgactttc	tggtctgtaa
751	ctgacactga	ggcgcgaaag	cgtggggagc	aaacaggatt	agataccctg
801	gtagtccacg	ccgtaaacga	tgagtgctaa	gtgttagagg	gtttccgccc
851	tttagtgctg	aagttaacgc	attaagcact	ccgcctgggg	agtacggccg
901	caaggctgaa	actcaaagga	attgacgggg	gcccgcacaa	gcggtggagc
951	atgtggttta	attcgaagca	acgcgaagaa	ccttaccagg	tcttgacatc
1001	gtctgaaaac	yctagagata	grgcttctcc	ttcgggagca	gagtgacagg
1051	tggtgcatgg	ttgtcgtcag	ctcgtgtcgt	gagatgttgg	gttaagtccc
1101	gcaacgagcg	caacccttga	tcttagttgc	catcattagg	ttgggcactc
1151	taaggtgact	gccggtgaca	aacoggagga	aggtggggat	gacgtcaaat
1201	catcatgccc	cttatgacct	gggctacaca	cgtgctacaa	tggacggtac
1251	aaagagctgc	aagaccgcga	ggtggagcta	atctcataaa	accgttctca
1301	gttcggattg	taggctgcaa	ctcgcctaca	tgaagctgga	atcgctagta
1351	atcgcggatc	agcatgccgc	ggtgaatacg	ttcccgggcc	ttgtacacac
1401	cgcccgtcac	accacgagag	tttgtaacac	ccgaagtcgg	tggggtaacc
1451	tttatggagc	cagccgccta	aggtgggaca	gatgattggg	gtgaagtcgt
1501	aacaaggtag				

Figure 2. The 16 S rDNA nucleotide sequence of An 3 strain.

Table 6. Homology search to MicroSeq Bacterial Full gene Library using BLAST.

Name of entry	Identity (%)
Bacillus thuringiensis	99.47
Bacillus cereus	99.40
Bacillus mycoides	99.34
Bacillus flexus	91.93
Bacillus cohnii	90.87
Bacillus oleronius	90.81
Bacillus atrophaeus	90.61
Bacillus megaterium	90.34
Bacillus horikoshii	90.34
Bacillus mojavensis	90.15

influenced by the presence of Al, especially at a concentration of 200 ppm. This may be ascribed to Al's toxic effect at high concentration to the bacterium. In order to deal with the toxicity, some microorganisms have developed mechanisms to tolerate metal-stress conditions. Mechanisms for metal detoxification include export, chelation and metabolism. The export and metabolism of Al have not been reported, while the tolerance of plants to Al is related to the secretion of organic acids, which chelate inorganic monomeric Al (Kochian, 1995). Additionally, acid- and Al- tolerant root nodule bacteria produce a larger amount of exopolysaccharides (EPS) than sensitive strains under stress (Appanna, 1988). It has been indicated that the production of EPS is a strategy to neutralize the toxic effects of Al (Appanna, 1989), since an EPS capable of chelating Al may substantially decrease the activity of toxic ions on the cell surface (Cunningham and Munns, 1984). Besides, it has been suggested that the acid- and Al- tolerant isolate, *Flavobacterium* sp. ST-3991, released certain substances, perhaps protein and chelators, during

Table 7. Homology search to international nucleotide sequence database using BLAST.

Name of entry	Name of strain	Accession no.	Identity
Bacillus cereus	G8639	AY138271	1507/ 1512= 99.7%
Bacillus cereus	H1439	AY138270	1509/ 1512= 99.8%
Bacillus cereus	G9667	AY138273	1506/1512= 99.6%
Bacillus cereus	2000031486	AY138272	1506/1512= 99.6%
Bacillus thuringiensis	2000031482	AY138290	1504/1512= 99.5%

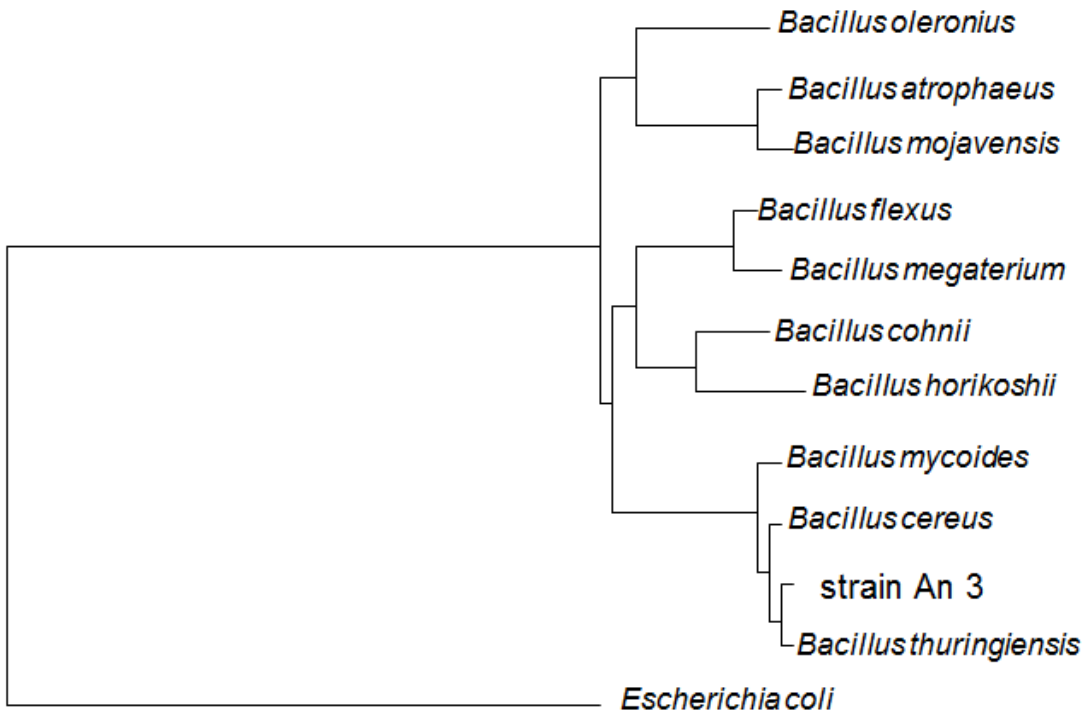

Figure 3. The Neighbor-joining molecular phylogenetic tree of strain An 3.

Table 8. Tolerance to acidity and Al of *Bacillus* sp. An 3.

pH	Al concentration (g L^{-1})						
	0	0.1	0.5	1.0	5.0	10.0	50.0
3.5	+	+	+	+	+	+	-
3.0	+	+	+	+	+	+	-
2.5	+	+	+	+	+	+	-
2.0	+	+	+	+	+	+	-

its growth, which might mask ionic Al and increase the pH of the medium. The masked Al appeared to form Al complexes because the culture medium became turbid and very viscous during the growth (Konishi et al., 1994). However, in the present study, during the growth of *Bacillus* sp. An 3, the pH of the medium without Al increased steeply and was neutral after 10 days, but that of the culture medium with Al was almost gradually decreased. This difference suggested the existence of a mechanism of responding to an increasing concentration of Al in the culture medium. However, elucidation of the precise mechanisms requires further study.

From acidic tea soils in Kagoshima in Japan, an acidity-tolerant and Al- resistant bacterium was isolated. The

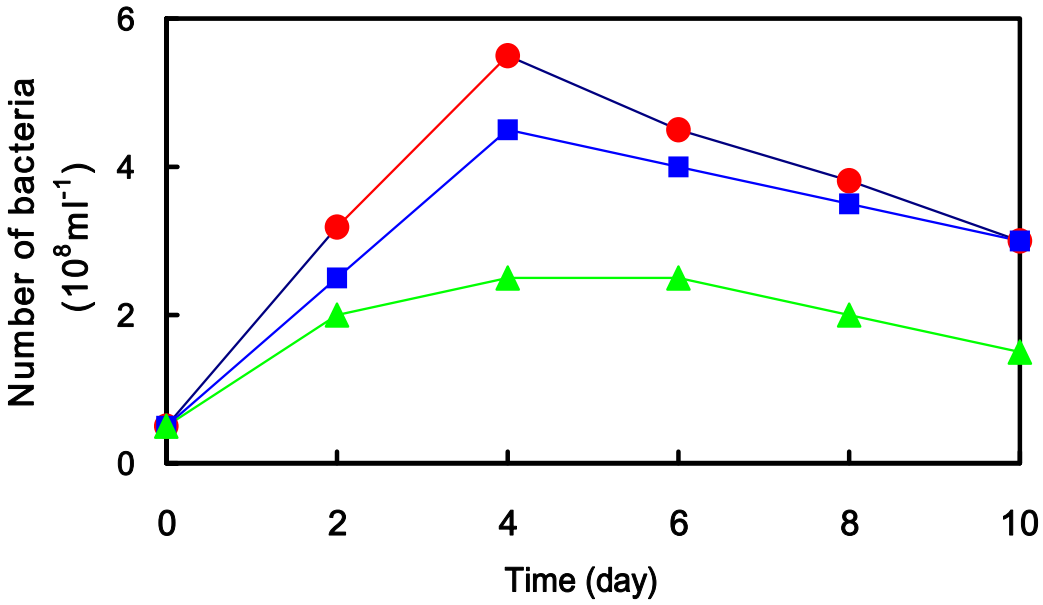

Figure 4. Growth of strain *Bacillus* sp. An 3 in LB liquid medium (pH 3.5) without Al (●), 100 ppm Al (■) and 200 ppm Al (▲).

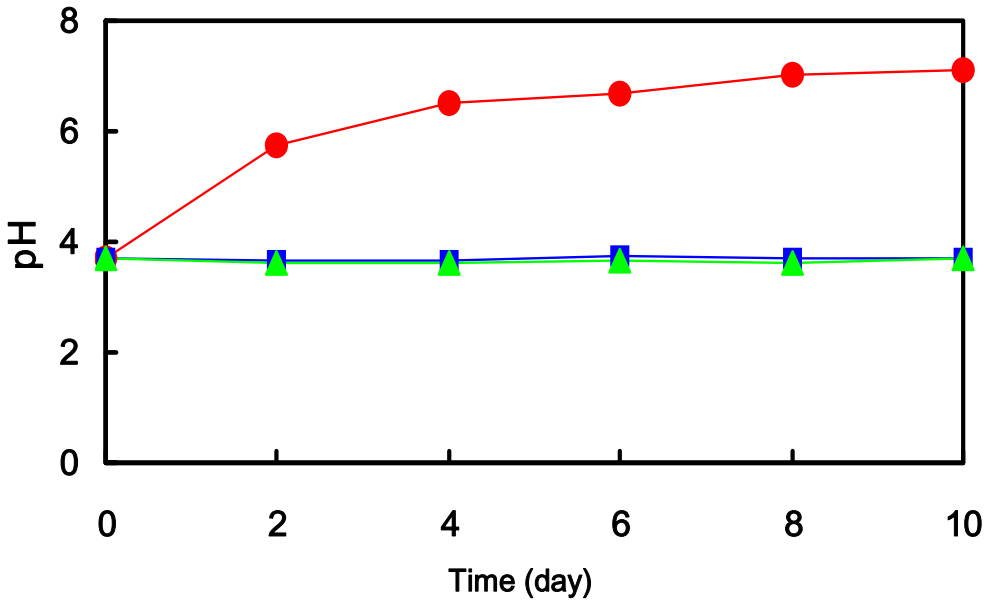

Figure 5. Changes of pH in response to increasing concentrations of Al in LB liquid medium (pH 3.5) without Al (●), 100 ppm Al (■) and 200 ppm Al (▲) of strain *Bacillus* sp. An 3.

isolate was identified as *Bacillus* sp. (with accession number DQ234657) and related to *B. cereus* and shown to tolerate high acidity, and to resist and eliminate a substantial amount of Al from the culture medium. These results not only have contributed to clarifying the characteristics of microbial ecology in acidified tea garden soil but also may facilitate studies on utilizing indigenous microorganisms to improve the current condition of tea garden soils.

Conflicts of Interest

The authors declare that they have no conflict of interest.

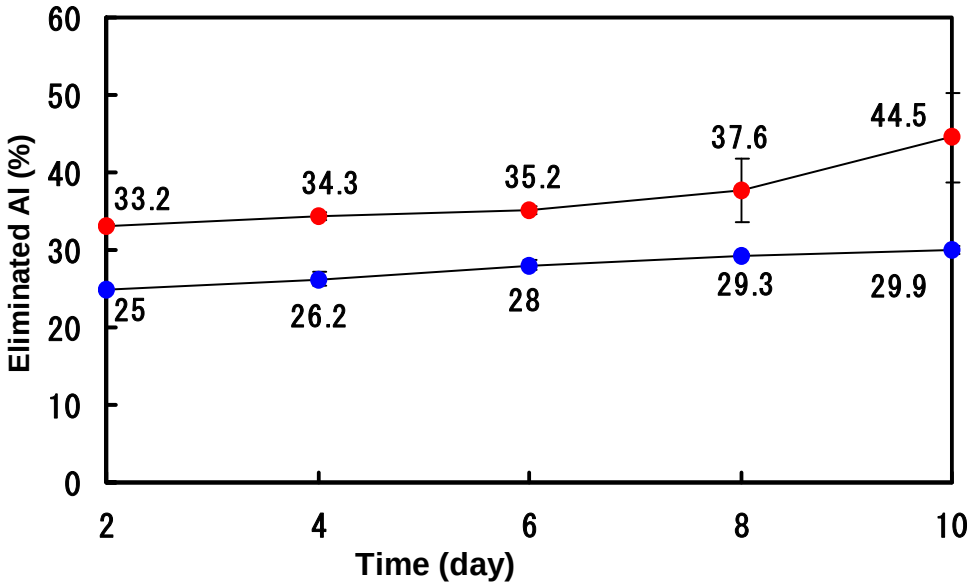

Figure 6. Elimination Al from LB medium (pH 3.5) containing 100 ppm Al (●) and 200 ppm Al (●) by strain *Bacillus* sp. An 3.

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