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Isolation of Al-tolerant yeasts and identification of their Al-tolerance characteristics

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Four strains of aluminium (Al) tolerant yeasts were isolated from a tea field in Yunnan province in China. The isolated yeast strains were able to grow on S-GM solid media supplemented with 100 to 200 mM Al^{3+}. Based on morphology and the sequences of the D1/D2 region of the 26S rDNA and the ITS region, the four strains were identified as *Cryptococcus humicola*, *C. rajasthanensis*, *C. laurentii* and *Rhodotorula mucilaginosa*. This is the first demonstration that *C. rajasthanensis*, *C. laurentii* and *R. mucilaginosa* are able to grow in the presence of Al. Growth of *C. humicola* and *C. rajasthanensis* was inhibited when they were grown in broth containing 100 mM Al^{3+}, and growth of *C. laurentii* and *R. mucilaginosa* was inhibited when they were grown in broth containing 50 mM Al^{3+}. The residual amount of inorganic monomeric Al in the media after culture with three of the strains (*C. humicola*, *C. rajasthanensis* and *C. laurentii*) was decreased, but the total amount of Al remained unchanged for all strains. These data suggested that they possessed different mechanisms of Al tolerance. These highly Al-tolerant microbial strains are suitable for future studies to elucidate the mechanisms of Al tolerance.

Key words: Aluminium, *Cryptococcus humicola*, *C. rajasthanensis*, *C. laurentii*, *Rhodotorula mucilaginosa*.

INTRODUCTION

Aluminium (Al) is the most abundant metal in the earth's crust (Cox, 1995). It is present in an insoluble form under neutral and alkaline conditions, but becomes soluble Al^{3+} as acidity increases. The form of Al^{3+} is toxic to animals, plants and microbes. Al exerts its toxic effects on microbes by competing with magnesium and iron (Macdonald & Martin, 1988; Illmer & Buttinger, 2006), and by binding to DNA, membranes or cell walls (Pina & Cervantes, 1996).

Research on Al toxicity and Al resistance mechanisms has primarily focused on higher plants. Two types of Al-tolerant mechanisms have been reported. Internal detoxification of Al depends on low molecular weight ligands, such as organic acids or Al-binding proteins binding Al in the cytoplasm, or sequestrating Al within an internal compartment. Al resistant plants with external detoxification mechanisms can release organic acids that chelate Al, such as citrate, malate and oxalate (Pellet *et al*., 1996; Ryan *et al*., 2001), into the rhizosphere. Additionally, the permeability of the plasma membrane decreases the influx of Al (Kochian, 1995). Some microbes have developed mechanisms similar to those used by higher plants to detoxify Al. *Pseudomonas fluorescens* can produce citrate (Mailloux *et al.*, 2008) and oxalate (Hamel *et al*., 1999; Appanna *et al*., 2003), which bind Al. Furthermore, phosphatidylethanolamine plays a vital role in the detoxification of Al in *P. fluorescens* (Hamel & Appanna, 2003).

Exopolysaccharides produced by *Bradyrhizobium* strains can precipitate Al at a pH of about 4.8 (Corzo *et al*., 1994). However, internal and external sequestration of Al is not the primary mechanism of Al tolerance used by *Penicillium janthineleum* (Zhang *et*

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al., 2002). An increased number of mitochondria and upregulation of mitochondrial genes are responsible for the adaptive increased resistance to Al by *Rhodotorula glutinis* (Tani *et al*., 2008). The study of the different Al-detoxification mechanisms in microbes and plants may potentially lead to the discovery of new mechanisms underlying Al toxicity and resistance. Therefore, we attempted to isolate microbial strains that were able to tolerate high levels of Al.

Many microbes including bacteria and fungi that were isolated from acidic soil are able to tolerate Al. Kawai *et al*. (2000) found six strains that were able to tolerate up to 100-200 mM of Al in acidic conditions and identified them as *Cryptococcus humicola*, *R. glutinis*, *Aspergillus flavus*, *Penicillium* sp., *P. janthinellum* and *Trichoderma asperellum*. *Aspergillus flavus*, *Penicillium* sp. and *P. janthinellum* were able to decrease the amount of toxic inorganic monomeric Al in the glucose medium. All strains were able to remove Al from the soil extract medium. Kanazawa *et al*. (2005) isolated 38 strains of acid-tolerant microorganisms from acidic soil, where tea is grown, that could grow strongly in YG media containing 100 mM Al^{3+} at pH 3. These strains belong to two different yeast species, *Cryptococcus* sp. and *Candida palmioleophila*, as determined by their 28S rDNA-D1/D2 sequences. These strains were also able to eliminate Al from the culture media. Travis (1998) reported a bacterium whose growth depended on a sufficient amount of Al. Aizawa *et al*. (2010) isolated two strains of Al-tolerant bacteria from a Chinese water chestnut that was growing in a highly acidic swamp (pH 2-4) with acid sulphate in the soil and identified them as members of the genus *Burkholderia*. Kimoto *et al.* (2009) isolated an Al-tolerant bacterium from a waterweed growing in a highly acidic swamp (pH 3) in an area with acid sulphate in the soil and classified it as belonging to the genus *Acidocella*, class *Alphaproteobacteria*. Micromolar concentrations of Al can severely inhibit plant growth; however, these Al-tolerant strains can tolerate Al concentrations as high as 200 mM. The mechanism responsible for this extremely high tolerance of Al is unknown.

In the present study, we reported the isolation and characterisation of Al-tolerant yeasts that were isolated from the soil of a tea garden. We determined whether these yeasts were able to tolerate Al and examined the total amount of Al and residual inorganic monomeric Al that remained in the spent culture media. These microbial strains that are highly tolerant to Al can be used for further research to elucidate their Al-tolerance mechanisms.

MATERIALS AND METHODS

Properties of the soil samples

The soil samples were collected at a depth of 5-20 cm from tea fields in Yunnan Province, China. All of the fresh soil samples were kept in sterile plastic bags and stored at 4°C. The moisture content and the pH of the water solution from the soil samples were analysed, according to the procedure of Kanazawa *et al.* (2005) with some modifications. Soil samples were heated at 105°C for 24 hrs to remove excess moisture. The moisture content was expressed as the percentage of water collected compared to the total weight of the sample. The water soluble Al in soil was extracted at a 1:10 (w/v) ratio with deionised water. The Al content was quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis, and the pH of the water solution was determined using a glass electrode (Kanazawa *et al*., 2005).

Isolation of Al-tolerant microorganisms

S-LB broth and S-GM media were used to screen Al-tolerant microorganisms according to Kawai *et al*. (2000). Al-tolerant microorganisms were enriched in the S-LB broth (pH 3.0) supplemented with 5 mM Al^{3+}. These microorganisms were then cultivated on S-GM media (pH 3.0) containing 5 mM Al^{3+} at 30°C for 3-10 days, at which point, microbial colonies were observable. Ten Al-tolerant colonies were selected and then purified on S-GM media with higher concentrations of Al^{3+}.

DNA preparation and analysis of the D1/D2 region and the ITS region

Total genomic DNA was extracted and purified according to the protocol described in Tapia-Tussell *et al*. (2006). The identity of the isolated strains was determined by colony morphology, microscopic examination and sequence analysis of the D1/D2 region of the 26S rDNA and the ITS region. The D1/D2 region of the 26S rDNA was amplified using the universal primers NL_1 (5′-GCATATCAATAAGCGAGAGAAAAG-3′) and NL_4 (5′-GGTCCGTGTTTCAAGACGG-3′). The ITS1-5.8S-ITS2 region was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Libkind *et al*., 2003). Amplification was

conducted under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were purified using the Tiangen PCR Purification Kit (Tiangen, China). Sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Sequences of the 26S rDNA (D1/D2 region) and the 5.8S-ITS region were compared to the nonredundant NCBI database by using BLASTN and deposited in the GenBank database under the accession numbers shown in Table 1.

Al-tolerance assays

Four strains with different morphological features were pre-incubated in the appropriate medium until they reached an OD_{600} of approximately 1.0. For the growth assays, the initial OD_{600} was adjusted to 0.05 and then the cells were incubated at 30°C while shaking at 200 rpm. The OD_{600} of the cultures was measured every 2 hrs using a spectrophotometer (SHIMADZU, UV-1700, Japan). For spot assays, the OD_{600} of each culture was adjusted to 2.0. Then, 10-fold serial dilutions were prepared (1:1, 1:10, 1:100, 1:1000 and 1:10000) and 6 μl of each dilution was spotted onto S-GM plates (pH 3.0) supplemented with 0, 0.1, 1, 20, 50, 100, 150 or 200 mM of Al^{3+}. Each sample was spotted in triplicate and three independent experiments were conducted.

Quantification of Al content in the culture medium

The cells were incubated overnight, transferred to new S-GM medium containing 20 mM Al^{3+}, and then the initial OD_{600} was adjusted to 0.1. After 24 hrs of incubation, the culture was centrifuged at 12000 rpm for 10 min and the supernatant was filtered using a sterilised filter with 0.2 μm pores. Then, the amount of inorganic monomeric Al and total Al in the filtered supernatant was determined. Inorganic monomeric Al was measured using the pyrocatechol violet method (Kerven *et al*., 1989). Total Al was measured using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Leeman, PS1000, USA). Briefly, 0.5 ml of chlorhydric acid was added to 2 ml of culture supernatant and adjusted to a volume of 25 ml with ultrapure water. The following conditions were used for ICP-AES: the radio-frequency generator was set at 1.0 kW, a plasma argon flow rate of 0.2-0.5 L min^{-1} and a cooling gas flow of 16 L min^{-1}. The analyses were performed in duplicate.

Statistical analysis

The data reported in this paper are mean values based on three replicates. Statistical analysis was conducted by the Statistical Product and Service Solutions (SPSS 11.5). Means were separated by analysis of variance (ANOVA) and the significant differences were assessed by Duncan's multiple range test at $p < 0.05$.

RESULTS

Soil properties and isolation of Al-tolerant microorganisms

The properties of the soil samples from Longling in Baoshan City of Yunnan province, which were designated No. 1, 2, and 3, were analysed. As shown in Table 2, the content of water soluble Al was the highest in soil sample No. 1.

Ten colonies that grew on the S-GM plate containing 5 mM Al^{3+} were isolated and purified. These colonies were considered to be Al tolerant. Four Al-tolerant strains with different morphology, designated BSLL1-1, BSLL1-28, BSLL3-4 and BSLL3-13, were then transferred to S-GM agar plates with higher concentrations of Al^{3+} to test their resistance to Al. BSLL1-1 and BSLL1-28 were isolated from soil sample No. 1, and BSLL3-4 and BSLL3-13 were isolated from soil sample No. 3.

TABLE 1. Blast results of sequences produced in this study (D1/D2 region of 26S rDNA and ITS)

Strains	Length (bp) 26S D1/D2	ITS	Homology to species	Identity 26S D1/D2	Identity ITS	Accession number 26S D1/D2	Accession number ITS
BSLL1-1	625	535	*Cryptococcus humicola*	100%	99%	HM459598	HM459599
BSLL1-28	640	537	*Cryptococcus rajasthanensis*	100%	100%	HM459600	HM459601
BSLL3-4	540	561	*Cryptococcus laurentii*	100%	100%	HM469460	HM469461
BSLL3-13	613	604	*Rhodotorula mucilaginosa*	99%	99%	HM469462	HM469463

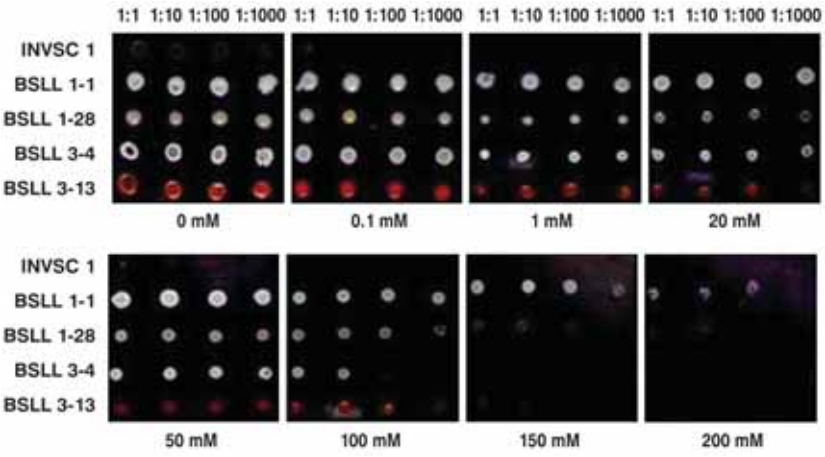

FIG. 1. Al tolerance of the isolated strains. BSLL1-1, BSLL1-28, BSLL3-4, BSLL3-13: Al-tolerant isolates; INVSC 1: Al-sensitive yeast strain. The initial OD_{600} of each culture was 2.0. The cultures were diluted in 1:1, 1:10, 1:100 and 1:1000, and then 6 µl of each dilution was spotted onto S-GM plates containing 0 to 200 mM Al^{3+}.

Al tolerance of the isolated microorganisms

To test the ability of the four Al-tolerant strains to grow in the presence of Al, the isolates were transferred to S-GM agar plates with higher concentrations of Al^{3+}. As shown in Figure 1, all isolates were able to tolerate very high concentrations of Al. BSLL3-4 and BSLL3-13 were able to grow in the presence of 100 mM of Al^{3+}. BSLL1-1 and BSLL1-28 were able to grow on media containing 200 mM of Al^{3+}. BSLL1-1 showed better growth than BSLL1-28 in the presence of 200 mM of Al^{3+}. Thus, these yeasts were able to tolerate inorganic monomeric Al up to 100-200 mM.

Morphology and phylogenetic relationship of the isolated microorganisms

Sequences analysis of the D1/D2 region of the 26S rDNA and the ITS region indicated that the isolates had the highest homologies to *C. humicola*, *C. rajasthanensis*, *C. laurentii* and *R. mucilaginosa* (Table 1).

Additionally, the morphological features of the colonies were examined under a light microscope. All four strains were found to be oval. BSLL1-1, BSLL1-28 and BSLL3-4 were 3-4.5 × 4.5-5 µm in size and had white colonies. BSLL3-13 was 3-3.5 × 4.5-6 µm in size and had red colonies. Thus, the four unknown Al-resistant strains were identified as *C. humicola* (BSLL1-1), *C. rajasthanensis* (BSLL1-28), *C. laurentii* (BSLL3-4) and *R. mucilaginosa* (BSLL3-13).

Growth of the isolated microorganisms at high concentrations of Al

The effects of Al on the growth of the isolates in broth medium were also examined. The growth of the four strains was not affected at low concentrations of Al (5 mM), but growth was inhibited when the organisms were cultured at high concentrations of Al (Fig. 2). The lag phase of all strains when cultured at high concentrations of Al occurred later and was prolonged compared with the culture with no Al. The growth of strains *C. humicola* (BSLL1-1) and *C. rajasthanensis* (BSLL1-28) was inhibited by 100 mM of Al^{3+}, whereas the growth of strains *C. laurentii* (BSLL3-4) and *R. mucilaginosa* (BSLL3-13) was inhibited when cultured at 50 mM and 20 mM of Al^{3+}, respectively. However, *C. rajasthanensis* (BSLL1-28), *C. laurenti* (BSLL3-4) and *R. mucilaginosa* (BSLL3-13) could not grow in media containing 150 mM or 200 mM Al^{3+} (Fig. 2A). The growth of BSLL3-13 under 20 mM Al stress was decreased greatly with the culture time and showed significant difference with that of other three strains ($p < 0.05$). At 50 mM Al concentration, the growth of BSLL1-28, BSLL3-4 and

TABLE 2. Properties of the soil samples collected from tea gardens (all values are the means of three replicates)

Soil samples	Depth (cm)	Moisture (%)	pH (H_2O)	Water soluble Al (µmol kg^{-1})
No. 1	5-20	17.6	6.25	34.7 ± 0.46
No. 2	5-20	60.4	6.50	24.3 ± 0.35
No. 3	5-20	53.8	5.56	29.6 ± 0.82

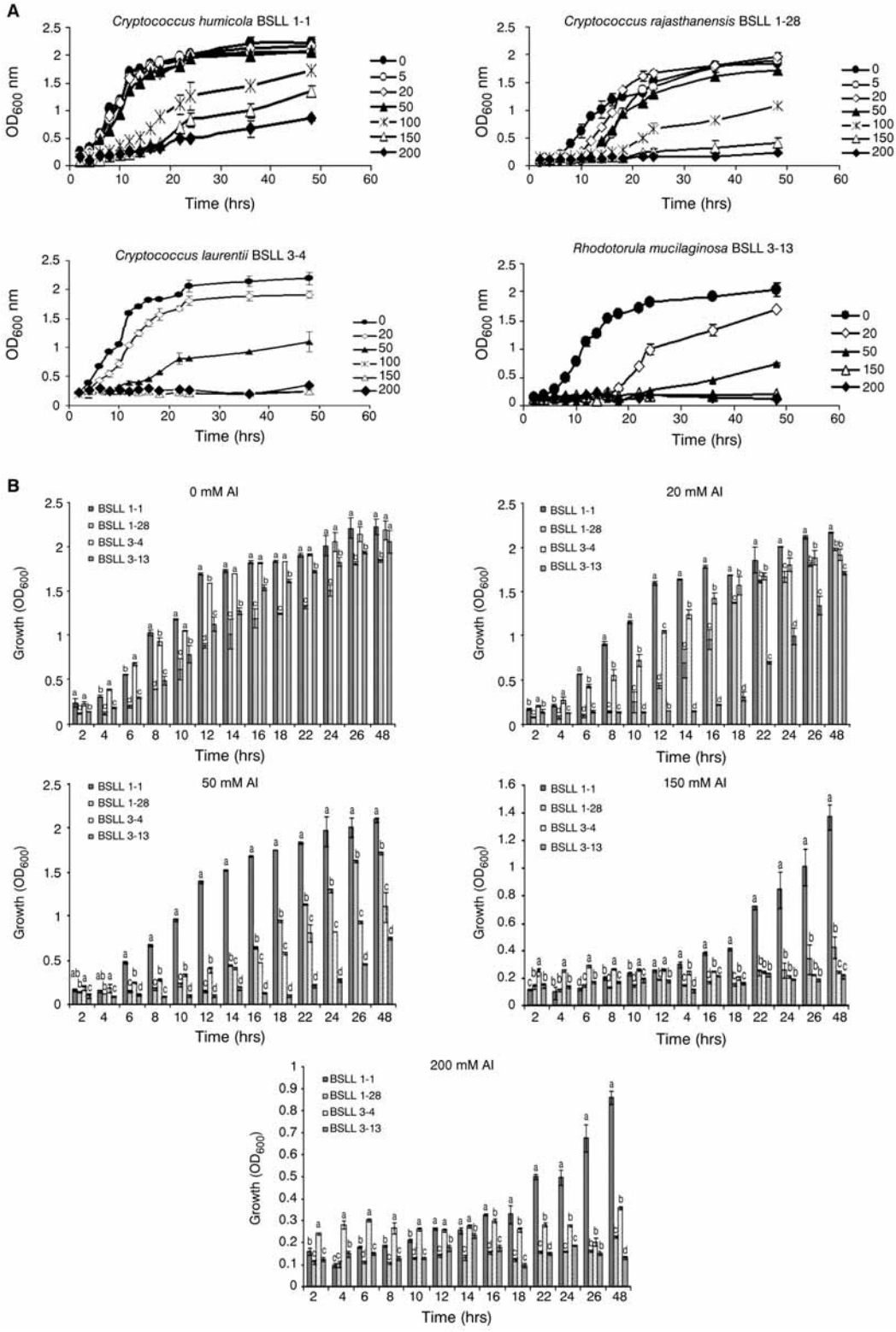

FIG. 2. Growth of the four Al-resistant strains at different concentrations of Al in S-GM broth medium, pH 3.0. The initial OD_{600} of each culture was adjusted to 0.05, and then the culture incubated at 30°C while shaking at 200 rpm. The OD_{600} was measured at different culture time. Growth curves of Al-resistant yeasts under Al stress (A). No Al (●), 5 mM (○), 20 mM (◇), 50 mM (▲), 100 mM (✳), 150 mM (△), 200 mM (♦). Bars represent means ± SD (n = 3). Comparisons of Al-tolerant abilities among four isolated yeasts at same time and same Al concentrations (B). Values are presented as the mean of three separate measurements. Bars represent means ± SD (n = 3). Different letters represent significant differences at $p < 0.05$. Same letters indicate no significant differences at $p < 0.05$.

TABLE 3. Residual inorganic monomeric Al and total Al in the culture supernatants (uninoculated media was shaken and used as a negative control; residual Al in negative control was designated as 100%; all values are means of three replicates; asterisk means significant difference compared with control at $p < 0.05$)

Strain	Final pH	Residual Al	
		Monomeric Al (%)	Total Al (%)
Blank	3.00	100	100
BSLL1-1	2.93	82 ± 1.26 *	99.7
BSLL1-28	2.96	94 ± 11.9	100.4
BSLL3-4	2.99	95 ± 6.1	100.1

BSLL3-13 decreased more significantly than that of BSLL1-1. Judged from the growths of 150 mM and 200 mM Al concentrations, BSLL1-1 was able to tolerate the highest levels of Al among four strains (Fig. 2B).

Elimination of Al from the culture media by the Al-resistant microorganisms

The culture supernatant was obtained by centrifuging at 12,000 rpm for 10 min. Inorganic monomeric Al and total Al were measured. When culture was performed in medium containing 20 mM of Al^{3+}, the pH of the spent medium decreased slightly compared to the initial pH of 3.0 (Table 3). The residual inorganic monomeric Al was decreased slightly in the spent media of *C. rajasthanensis* (BSLL1-28) and *C. laurentii* (BSLL3-4). In the spent media of *C. humicola* (BSLL1-1), the residual inorganic monomeric Al was decreased greatly and showed statistically significant difference compared with the control media. However, the total Al in the spent media from the four strains did not decrease.

DISCUSSION

Al exists as inorganic monomeric Al in acidic conditions, which is toxic to most organisms. Tea fields are usually acidic because ammonium sulphate is used as fertiliser (Watanabe & Ikegaya, 1987). Therefore, the soil in tea fields is often used to isolate Al-tolerant microorganisms. In previous reports, a number of acid-tolerant and Al-tolerant microorganisms were isolated from the soil of tea fields (Kanazawa & Kunito, 1996; Kawai *et al*., 2000; Kanazawa *et al*., 2005). In our research, soil samples were collected from tea fields. The samples were weakly acidic and contained micromolar levels of water soluble Al, which is harmful to plants.

Most of the Al-tolerant microorganisms that have been isolated from acidic soils are yeasts, such as *C. humicola*, *R. glutinis* and *C. palmioleophila*, and fungi, including *A. flavus*, *Penicillium* sp., *P. janthinellum*, *Emericellopsis* sp., *Paecilomyces lilacinus*, *Moritierella ramanniana* var. *angulispora*, *Sprothrix inflata*, *P. glabrum*, *Metarhizum anisopliae*, *Chaetospharia inaeqalis* and *A. fumiatus* (Kanazawa & Kunito, 1996; Kawai *et al*., 2000; Kanazawa *et al*., 2005). Recently, a few Al-tolerant bacteria were isolated (Kimoto *et al*., 2009; Aizawa *et al*., 2010). This may be because fungi and yeast are more tolerant of acidic conditions than bacteria. In the present study, all four isolated Al-tolerant strains are yeast. This is the first demonstration for *C. rajasthanensis*, *C. laurentii* and *R. mucilaginosa* to tolerate Al.

Micromolar concentrations of Al can severely inhibit plant growth (Barcelo & Poschenrieder, 2005). However, these Al-tolerant strains can tolerate much higher concentrations of Al than plants. This may suggest that they are able to adapt to conditions that are acidic and contain high concentrations of Al. It is possible that they have evolved special Al-tolerance mechanisms that are different from those in plants. *Cryptococcus humicola* (BSLL1-1) was able to tolerate the highest concentrations of Al of the four yeasts and originated from soil sample No. 1, which also had the highest content of water soluble Al.

Some microorganisms secrete organic acids that bind inorganic monomeric Al (Gadd, 1999; Hamel *et al*., 1999; Appanna *et al*., 2003; Hamel & Appanna, 2003). In this study, the concentration of inorganic monomeric Al decreased in the culture media of *C. humicola* (BSLL1-1), *C. rajasthanensis* (BSLL1-28) and *C. laurentii* (BSLL3-4). However, no change in the level of total Al was observed. These results indicated that the decrease in inorganic monomeric Al was not due to the uptake and accumulation of Al in the cells. However, the inorganic monomeric Al may be converted to the chelated form by organic acid in

the medium. *Rhodotorula mucilaginosa* (BSLL3-13) did not eliminate inorganic monomeric Al from the spent medium (data not shown). These results suggested that the Al-resistant fungi have developed different mechanisms to adapt to acidic and toxic Al conditions. Therefore, the mechanism of Al tolerance needs to be studied further. Previous studies on the mechanisms of Al tolerance used by microbes have mainly focused on *P. fluorescens* (Hamel *et al*., 1999; Singh *et al*., 2005; Mailloux *et al*., 2008), *Saccharomyces cerevisiae* (MacDiarmid & Gardner, 1998; Hamilton *et al*., 2001; Basu *et al*., 2004; Kakimoto *et al*., 2005) and *R. glutinis* (Tani *et al*., 2008, 2010). The mechanisms of Al tolerance of other soil microorganisms are still poorly understood. Therefore, these yeasts that are tolerant to high levels of Al are suitable for studying the molecular mechanisms of Al tolerance, cloning of new Al-tolerant genes and may potentially lead to the development of technologies that can be used for bio-remediation of acidic soil with a high Al content.

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