The crucial role of mitochondrial regulation in adaptive aluminium resistance in *Rhodotorula glutinis*

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*Rhodotorula glutinis* IFO1125 was found to acquire increased aluminium (Al) resistance from 50 µM to more than 5 mM by repetitive culturing with stepwise increases in Al concentration at pH 4.0. To investigate the mechanism underlying this novel phenomenon, wild-type and Al-resistant cells were compared. Neither cell type accumulated the free form of Al (Al³⁺) added to the medium. Transmission electron microscopic analyses revealed a greater number of mitochondria in resistant cells. The formation of small mitochondria with simplified cristae structures was observed in the wild-type strain grown in the presence of Al and in resistant cells grown in the absence of Al. Addition of Al to cells resulted in high mitochondrial membrane potential and concomitant generation of reactive oxygen species (ROS). Exposure to Al also resulted in elevated levels of oxidized proteins and oxidized lipids. Addition of the antioxidants α-tocopherol and ascorbic acid alleviated the Al toxicity, suggesting that ROS generation is the main cause of Al toxicity. Differential display analysis indicated upregulation of mitochondrial genes in the resistant cells. Resistant cells were found to have 2.5- to 3-fold more mitochondrial DNA (mtDNA) than the wild-type strain. Analysis of tricarboxylic acid cycle and respiratory-chain enzyme activities in wild-type and resistant cells revealed significantly reduced cytochrome c oxidase activity and resultant high ROS production in the latter cells. Taken together, these data suggest that the adaptive increased resistance to Al stress in resistant cells resulted from an increased number of mitochondria and increased mtDNA content, as a compensatory response to reduced respiratory activity caused by a deficiency in complex IV function.

**INTRODUCTION**

Aluminium (Al) is a light metal that makes up 7% of the earth’s crust and is the third most abundant element after oxygen and silicon (Ma et al., 2001). Al forms harmless silicates or hydroxide complexes at neutral pH. However, when soils become acidic as a result of natural processes or human activities, Al is solubilized into a toxic trivalent cation. Al toxicity has been recognized as a major factor limiting plant productivity in acidic soils. Al is also known as a potent neurotoxin in animal cells and its relevance to Alzheimer’s disease is hotly debated (Exley, 1999). Al can cause toxicity in micro-organisms as well (MacDiarmid & Gardner, 1996). Al toxicity induces programmed cell death in yeast (Zheng et al., 2007), plants (Yakimova et al., 2007) and animals (Kawahara, 2005). A significant amount of research has been conducted on mechanisms of Al toxicity and tolerance using *Saccharomyces cerevisiae* as a model micro-organism (Basu et al., 2004; Hamilton et al., 2001; Kakimoto et al., 2005; MacDiarmid & Gardner, 1998).

Little mechanistic information is available on acid- and Al-resistant soil micro-organisms, most of which comprise filamentous fungi and basidiomycetous yeasts. We have isolated resistant micro-organisms from acidic tea soil, which were resistant to as much as 100–200 mM Al (Kawai...
This high resistance seemed to be more than enough to allow survival in acidic soil, whereas micromolar concentrations of Al severely inhibit plant growth. While *Rhodotorula glutinis* strain Y-2a is one such tolerant soil microbe, the type strain of *R. glutinis* (IFO1125) was found to be sensitive to Al (Tani et al., 2004). To derive resistant cells from the wild-type IFO1125 strain, it was cultivated with repeated stepwise increases in Al concentration, which resulted in acquisition of a heritable resistance phenotype to an Al concentration of ~5 mM that was not lost by repetitive cultivation in the absence of Al (Tani et al., 2004). To our knowledge, this is the first report of microbial acclimation to increasing Al stress. In this study, we compared wild-type and resistant cells in order to determine the mechanisms responsible for adaptive Al resistance.

**METHODS**

**Yeast and bacterial strains and culture conditions.** *R. glutinis* strain IFO1125 (ATCC 2527) was used as a wild-type strain. Al-resistant mutants R1000, R2000, R3000 and R4000 were obtained by repeated culture of the wild-type strain in the presence of increasing Al concentration (in 50 μM increments from 0 to 1000 μM, in 100 μM increments from 1000 to 2000 μM, and in 250 μM increments from 2000 to 4000 μM) in synthetic medium (SM pH 4.0), as described by Tani et al. (2004). Resistant mutants were designated with the letter R followed by a number indicating the AlCl₃ concentration ([AlCl₃]) and used to culture of the wild-type strain in the presence of increasing Al concentration (in 50 μM increments from 0 to 1000 μM, in 100 μM increments from 1000 to 2000 μM, and in 250 μM increments from 2000 to 4000 μM) in synthetic medium (SM pH 4.0), as described by Tani et al. (2004). Resistant mutants were grown in medium with each maximal Al concentration a total of three times, after which the final culture was frozen. Resistant mutants were streaked from the frozen cultures onto solid SM without AlCl₃. Single-colony isolates were picked from a filter-sterilized 0.1 M AlCl₃ stock. A basal concentration of 50 μM (0.2 mM and 0.1 mM, respectively), and succinate (20 mM, pH 4.0) was added and the solution was incubated at 160 °C for 1–2 h to evaporate the nitric acid. The resulting solution was diluted appropriately with 0.1 M HCl and used to determine aluminium concentration. The Al concentration was determined with a polarized Zeeman atomic absorption spectrophotometer (Hitachi-Z2000).

**Aluminium measurement.** Exponential-phase cells were washed twice with 0.85% NaCl and suspended in 1 ml 0.85% NaCl. A portion of the cell suspension was used to determine c.f.u. on SM plates and 100 μl aliquots of suspension ([AlCl₃]) were dried at 90–95 °C. To the dried samples, 500 μl HNO₃/HSO₃ (1:1, v/v) was added and the solution was incubated at 160 °C for 1–2 h to evaporate the nitric acid. The resulting solution was diluted appropriately with 0.1 M HCl and used to determine aluminium content. The Al concentration was determined with a polarized Zeeman atomic absorption spectrophotometer (Hitachi-Z2000).

**Speciation of Al using ²⁷Al-NMR.** Wild-type and resistant cells were separated from cultures by centrifugation, and the supernatants (570 μl) were transferred to glass NMR tubes (5 mm diameter) and subjected to a liquid-state ²⁷Al-NMR analysis (JNM-600 FT-NMR system, JEOL). The experimental parameters were: frequency, 156.25 MHz; spectral width, 62.5 kHz; data size, 32k; number of scans, 1300–76000; repetition time, 0.924 s; temperature, 298 K. The standard chemical shift (0 p.p.m.) was adjusted externally using 2.5 mM AlCl₃ solution in 0.1 M HCl after shimming against D₂O (Hiradate et al., 1998).

**Transmission electron microscopy.** Cells were harvested, washed with 0.85% NaCl, and fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 2 h. The cells were then treated with 1% potassium permanganate for 16 h at 4 °C, after which they were washed with water and dehydrated using a series of ethanol solutions (50–100%). Finally, ethanol was replaced with acetone and the cells were embedded in Epon 812 resin (TAAB Laboratories Equipment) according to the manufacturer’s instructions. Ultrathin sections were cut with a diamond knife, stained with 1% uranyl acetate and Reynolds lead citrate, examined in a Hitachi model H-7100 transmission electron microscope, and photographed.

**Fluorescent microscopic analysis of mitochondrial membrane potential and reactive oxygen species (ROS) generation in the presence of Al.** Wild-type and R2000 cells were grown in SM in the absence of Al. When the OD₆₀₀ reached 1.0, Al was added (50 μM for the wild-type and 2000 μM for R2000), and incubation was continued. Samples were withdrawn and cells were washed twice with 0.85% NaCl, and stained with 200 nM 3,3′-dihexyloxacarbocyanine iodide [DiOC₆(3)], Molecular Probes) and 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes) to assess mitochondrial membrane potential and ROS generation, respectively. The same procedure was also applied to exponentially growing cells in Al-supplemented media.

Stained cells were observed using a fluorescence microscope equipped with a 100 W Hg lamp (BX50 Olympus microscope) and charge-coupled device (CCD) images were taken with an Olympus DP70 digital camera. An excitation wavelength of 470–490 nm was used for DiOC₆(3) and H₂DCFDA and the resulting images were collected using a 510–550 nm band-pass filter.

**Subcellular fractionation.** Cells grown with and without Al were washed twice with 0.85% NaCl and resuspended in Tris/HCl (50 mM, pH 8.0). The resuspended cells were lysed in five 30 s pulses using a Mini-head beater (Wako Chemicals), followed by centrifugation at 2000 g for 10 min at 4 °C to remove unbroken cells. The supernatant was then centrifuged at 160 000 g for 10 min at 4 °C. The pellet was suspended in 50 mM Tris/HCl (pH 8.0) containing 0.5% n-dodecyl-β-maltopyranoside and designated the ‘membrane fraction’. The resulting supernatant was used as the ‘soluble fraction’. The membrane fraction was assayed for respiratory-chain activity, and the soluble fraction for TCA-cycle enzyme activities and oxidized proteins.

**Oxidized protein and lipid analysis.** The thiobarbituric acid-reactive species (TBARS) assay was used to measure oxidized membrane lipids, as described by Aydin et al. (2005). The washed cell suspensions were used to determine c.f.u. served as samples. Protein carbonyl content of soluble cell fractions was determined using the dinitrophenyl hydrazine (DPNH) assay (Frank et al., 2000). Protein concentration was determined using BSA as a standard (protein assay kit, Bio-Rad Laboratories).

**Molecular cloning.** Standard protocols were used for DNA cloning and transformation (Sambrook et al., 1989). Restriction enzymes and other DNA-modifying enzymes were purchased from TOYOBO. PCR was performed using ExTaq DNA polymerase (Takara Shuzo). DNA purification from agarose gels was done with MagExtractor (TOYOBO). PCR products were cloned into a pGEM-T easy vector
Cloning of partial mitochondrial DNA (mtDNA) from wild-type and R4000 strains. DNA libraries were constructed with BclI-digested genomic DNA of the wild-type and R4000 strains and EcoRI-digested pBluescript SK+ (Stratagene). The plasmids containing 9.8 kb BclI fragments from the wild-type and R4000 strains (pMT9kW and pMT9kR4, respectively) were then screened by colony hybridization (Sakai et al., 1999), using a COX3 DNA fragment (Table 2) as a probe. DNA from hybridizing plasmids was subcloned and sequenced. Gap closing was done by primer-walking.

PCR cloning of the actin gene. As a standard for real-time PCR to determine mtDNA copy number, an actin gene fragment was amplified from genomic DNA of the wild-type strain. Amino acid sequences of actin genes from various organisms [S. cerevisiae (YFL039C), Arabidopsis thaliana (At5g09810) and human (10120)] were selected and aligned by CLUSTAL W (http://align.genome.jp/).

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
</tr>
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<tr>
<td>A1</td>
<td>AATCTAGAGCTCCTCCTC</td>
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<tr>
<td>A2</td>
<td>AATCTAGAGCTCCACAGC</td>
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<tr>
<td>A3</td>
<td>AATCTAGAGCTCCTCGG</td>
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<td>A4</td>
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<td>A5</td>
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<td>B1</td>
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<tr>
<td>B2</td>
<td>CCTACACCGGTATATCC</td>
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<tr>
<td>B3</td>
<td>CATACACCGGATATGG</td>
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<tr>
<td>B4</td>
<td>ACCGCAACCGCAGAGG</td>
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<tr>
<td>B5</td>
<td>CACACGGCACAGCAAGA</td>
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<tr>
<td>C1</td>
<td>CATGTGTAACGCGTGGG</td>
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<tr>
<td>C2</td>
<td>CTTGTACATACAGTAAC</td>
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<tr>
<td>C3</td>
<td>CCATGGCGCATGAGA</td>
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<tr>
<td>C4</td>
<td>CCACACGCGCCACGGGA</td>
</tr>
<tr>
<td>C5</td>
<td>CCGCACGCACCGCAAGG</td>
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<tr>
<td>Ractin-F</td>
<td>GT(T/C/G)CT(T/C/G)GA(T/G)TC (G/C)GG(G/C)AT(G/T)GG</td>
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<tr>
<td>Ractin-R</td>
<td>AG(A/G)ATG(C/G)GA(G/T)CC(G/T)CC (A/G)ATTC</td>
</tr>
<tr>
<td>Rtime cox3-F</td>
<td>TGTGATAACCGGATGATAACAGAG</td>
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<tr>
<td>Rtime cox3-R</td>
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<tr>
<td>Rtime actin-F</td>
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<tr>
<td>Rtime actin-R</td>
<td>TTTGGCTACAGGTCTTCCTC</td>
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</table>

Two conserved regions (VLDSGDGV and WIGGSILASL) were found and used to design degenerate primers (Ractin-F and Ractin-R, respectively; Table 1). A partial actin gene (670 bp) was amplified by PCR from R. glutinis genomic DNA using the above primers, and was cloned into a pGEM-T easy vector (pGEM-Ractin), and sequenced.

Quantification of mtDNA copy number by real-time PCR. Primers for real-time PCR were synthesized (Rtime cox3-F and Rtime cox3-R for mtDNA, and Rtime actin-F and Rtime actin-R for the actin gene, Table 1) based on sequences of partial mtDNA and the actin gene, using Primer Express software (Applied Biosystems). mtDNA-encoded COX3 was selected for quantification. Both sets of primers for mtDNA and the actin gene were expected to yield 71 bp PCR fragments. mtDNA was quantified using the Applied Biosystems 7500 Realtime PCR system and SYBR Premix Ex Taq (Perfect Real-time, Takara). PCRs were performed in a total volume of 50 μl containing 10 pmol of each primer, 1 μl ROX II dye, 25 μl Premix and 0.2 μg total DNA. PCR cycling conditions were: initial denaturation at 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 1 min. Standard curves were created by analysing serial dilutions of cloned mtDNA (pMT9kW and actin gene (pGEM-Ractin). These plasmid solutions (0.045 pmol μl−1) were serially diluted 10-fold to generate 10 data points. The mtDNA content in total DNA from wild-type and resistant cells was normalized to the amount of actin DNA in each sample. PCR assays were performed in triplicate for each DNA sample. Genomic DNA isolated from wild-type and resistant cells grown in SM with and without AlCl3 were used as DNA templates.

Enzyme assays. Isocitrate dehydrogenase (Cook & Sanwal, 1969), α-ketoglutarate dehydrogenase (Nichols et al., 1994) and malate dehydrogenase (Englard & Siegel, 1969) activities in the soluble fractions were assayed as NADH-producing steps in the TCA cycle. NADH dehydrogenase (complex I) (Fang & Beattie, 2003), cytochrome oxidase (complex IV) (Wang et al., 2004) and ATP synthase (complex V) (Kagawa & Yoshida, 1979) were assayed in the membrane fraction. Citrate synthase (Sere, 1969), aconitase (Fasler & Lowenstein, 1969), succinate dehydrogenase (Bower, 1955; Fang et al., 2001; Samokhvalov et al., 2004; Oyedotun & Lemire, 2001) and fumarase (Hill & Bradshaw, 1969) were also assayed according to the references but their activities were not detectable by these methods.

Quantification of ATP. Wild-type and R4000 cells were collected, and washed three times with 0.85 % NaCl. Cell suspensions were then serially diluted (10-fold) and 25 μl of each suspension was mixed with 25 μl of the BacTiter-Glo Microbial Cell Viability Assay (Promega) reaction buffer. Luminescence was quantified using a MiniLumat (EG&G Berthold) for 10 s. ATP solutions (10 μM–1 mM) were used as standards. Experiments were done in triplicate. To calculate ATP content per cell, cell suspensions were spread on YPD plates and c.f.u. were determined after 3–5 days incubation at 28 °C. Mean c.f.u. were then used to calculate ATP content per cell.

Nucleotide sequence accession numbers The DNA sequences reported herein have been submitted to the DDBJ database under accession numbers AB248915 (partial mtDNA) and AB248916 (partial actin gene).

RESULTS AND DISCUSSION

Cellular Al content and chemical form of Al in growth media

Wild-type and R4000 cells were grown in the absence (control) or presence of Al (50 μM for the wild-type and...
2000 μM for R4000). Al-treated cells were found to contain more Al than control cells, but the amount of Al accumulated in R4000 was only four times higher than that in wild-type cells (Fig. 1a). This result suggested that resistant cells did not incorporate significant amounts of Al.

To elucidate the chemical form of Al in SM, $^{27}$Al-NMR spectra were acquired (Fig. 1b). No distinct shift in Al signal was found either before or after growth of the wild-type and resistant cells, suggesting that the major form of Al was $\text{Al}^{3+}$. A faint peak observed at about $-3.2$ p.p.m. may be due to Al chelated with sulfate ions (Hiradate, 2004). With decreasing pH values, the $^{27}$Al-NMR peak became sharp and shifted toward 0 p.p.m. This phenomenon corresponds to the increasing proportion of a symmetrical hexaaquaaluminium ion $\text{Al(H}_2\text{O)}_6^{3+}$, which gives a very sharp resonance peak (2 Hz) at 0 p.p.m., to an asymmetrical $\text{Al(H}_2\text{O)}_2^{3+}(\text{OH})^{2+}$, which gives a broader line width ($792 \pm 18$ Hz) at $3.5 \pm 1.3$ p.p.m. (Hiradate, 2004). This result suggests that Al resistance in $R$. glutinis is not conferred by inactivation of free Al ions by excreted chelators, as reported for fungi and plants (Ma et al., 2001; Kobayashi et al., 2004; Kawai et al., 2000).

**Physiological changes in mitochondria**

**Cellular and mitochondrial morphology affected by Al.**

The intracellular morphology of wild-type and resistant cells was compared by transmission electron microscopy.

### Table 2. Genes identified by DD analysis

<table>
<thead>
<tr>
<th>Related to</th>
<th>Gene</th>
<th>Wild-type*</th>
<th>R1000*</th>
<th>Size† (kb)</th>
<th>Primer‡</th>
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<td>Mitochondrial function</td>
<td>Cytochrome c oxidase subunit 1 (COX1)</td>
<td>-Al 1 +Al 1</td>
<td>-Al 3 +Al 3</td>
<td>0.4 A1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome c oxidase subunit 3 (COX3)</td>
<td>-Al 1 +Al 1</td>
<td>-Al 2 +Al 2</td>
<td>0.3 B2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADH dehydrogenase subunit 3 (NAD3)</td>
<td>-Al 1 +Al 1</td>
<td>-Al 2 +Al 2</td>
<td>0.2 C1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADH dehydrogenase subunit 5 (NAD5)</td>
<td>-Al 2 +Al 2</td>
<td>-Al 3 +Al 3</td>
<td>0.7 B5</td>
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<tr>
<td></td>
<td>Carbamoyl phosphate synthetase</td>
<td>-Al 1 +Al 1</td>
<td>-Al 2 +Al 2</td>
<td>1.0 B1</td>
<td></td>
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<tr>
<td></td>
<td>Tar1p (mitochondrial protein of unknown function)</td>
<td>-Al 1 +Al 1</td>
<td>-Al 2 +Al 2</td>
<td>1.0 A1</td>
<td></td>
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<tr>
<td>Membrane</td>
<td>Arsenite-translocating ATPase</td>
<td>-Al 4 +Al 1</td>
<td>-Al 1 +Al 1</td>
<td>1.0 C5</td>
<td></td>
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<td></td>
<td>Laccase</td>
<td>-Al 1 +Al 1</td>
<td>-Al 2 +Al 2</td>
<td>0.8 C4</td>
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<td>Signal transduction</td>
<td>$\text{Ca}^{2+}$/calmodulin-dependent protein kinase</td>
<td>-Al 4 +Al 1</td>
<td>-Al 1 +Al 1</td>
<td>0.5 B5</td>
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<td>Others§</td>
<td>Hypothetical proteins</td>
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*Numbers represent the intensity of the bands detected in DD: 1, not detected; 2, faint band; 3, moderate; 4, strong. Underlining indicates cloned fragment by DD.

†Detected band sizes in DD.

‡Primer names used in DD.

§Several other genes were homologous to various hypothetical proteins, and are not shown in the table.

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**Fig. 1.** (a) Cellular Al content determined by atomic absorption spectrometry. The wild-type and R4000 were grown in the presence of 50 μM and 2000 μM Al, respectively. The bars represent the mean ± SD ($n=3$). (b) Liquid-state $^{27}$Al-NMR spectra of the medium before and after cultivation of wild-type and R2000. The pH of the medium and concentrations of $\text{AlCl}_3$ used for cultivations are indicated in the figure. The media from R1000 and R4000 gave results similar to R2000.
(Fig. 2). Smaller mitochondria, with undeveloped cristae structures, were observed in wild-type cells grown in the presence of Al as compared to those grown in the absence of Al. R2000 cells had larger numbers of mitochondria than wild-type cells in general, but their mitochondria were smaller and less developed in the absence of Al, in contrast to wild-type cells.

**Increased mitochondrial membrane potential and concomitant ROS generation as a cause of Al toxicity.**

As shown in Fig. 3(a), addition of Al to cells from exponential-phase cultures grown in the absence of Al caused high mitochondrial membrane potential and concomitant ROS generation. The resistant strain exhibited higher mitochondrial membrane potential and ROS generation than the wild-type strain, even in the absence of Al.

Then we determined the mitochondrial membrane potential and ROS generation in cells grown in the presence of Al. Wild-type cells exhibited negligible ROS production under Al stress, while mitochondrial membrane potential was high, suggesting that the cells must have adapted to the Al by reducing ROS production (Fig. 3b). In contrast, resistant cells exhibited high ROS levels and mitochondrial membrane potential under Al stress, suggesting an acquired tolerance for high ROS and membrane potential.

As shown in Fig. 4(a), the growth of wild-type and resistant cells in the presence of Al resulted in increased levels of oxidized lipids and proteins. Lipids appeared to be targeted more than proteins by ROS attack. In addition, supplementation of α-tocopherol and ascorbic acid alleviated Al toxicity (Fig. 4b). Thus, ROS generation and concomitant oxidation of cellular components were considered to be major causes of Al toxicity.

**Differential expression of genes related to Al resistance**

**Identification of genes differentially expressed in wild-type and Al-resistant cells.** DD analysis was performed to identify differentially expressed genes in the wild-type and R1000 strains. RAP-PCR patterns for the two strains exhibited significant differences (data not shown). Differentially amplified DNA bands were isolated and sequenced, as shown in Table 2. Genes required for mitochondrial respiration (COX1, COX3, NAD3 and NAD5) were found to be upregulated in resistant cells.

**Increased mtDNA copy number in resistant cells.** mtDNA copy number in wild-type and resistant cells was analysed by real-time PCR (Fig. 5). The dissociation curves for each real-time PCR product showed that the PCR proceeded correctly without any by-product formation (data not shown). The mtDNA copy number in the wild-type strain was about 100 copies per cell and did not change in response to either the presence or absence of Al in early exponential phase. mtDNA copy number in the

![Fig. 2](http://mic.sgmjournals.org)

**Fig. 2.** Transmission electron microscopic analysis of wild-type and R2000 grown in the absence or presence of Al. Right, enlarged images of dotted squares to show morphology of mitochondria.
Fig. 3. (a) Effect of Al added to early-exponential phase cells (OD_{600} 1.0), grown under no Al stress, on mitochondrial membrane potential and ROS generation. Al was added to the cultures of wild-type and R4000 cells at their exponential phase grown in the absence of Al, and sampled at intervals to stain with DiOC6(3) and H2DCFDA. To compare fluorescence intensities, exposure time was set to 0.25 s for all samples. See Methods for detailed experimental procedure. Bar, 10 μm. (b) Effect of Al on the mitochondrial membrane potential and ROS generation in early-exponential-phase cells grown under Al stress.

Fig. 4. (a) Quantification of oxidized lipid and oxidized protein, shown as malondialdehyde equivalents and carbonyl equivalents, respectively. Wild-type and R4000 were grown in the presence of 50 μM and 2000 μM Al, respectively. The bars represent the mean ± SD (n = 3). (b) Effect of α-tocopherol and ascorbic acid on growth in the presence of Al. Wild-type (upper panels) and R4000 (lower panels) were grown in the presence of 50 μM and 2000 μM, respectively. Open symbols, without Al; filled symbols, with Al; squares, without antioxidants; circles, with antioxidants.
resistant cells in the presence of Al was 2.5–3.0-fold higher than in wild-type cells, but decreased to 1.2–2.1-fold in the absence of Al.

Al resistance and mitochondrial activity

Mitochondrial protein synthesis is important for growth under Al stress. Chloramphenicol binds to the mitochondrial ribosome, which leads to inhibition of mitochondrial protein synthesis. Chloramphenicol (100 µM) did not inhibit growth of wild-type or R4000 cells in the absence of Al, but it retarded their growth in the presence of Al (Fig. 6). This result suggested that ribosomal activity (namely mRNA translation) in mitochondria was important for growth in the presence of Al. This result also suggested that a functional mitochondrial respiratory chain, some of whose proteins are encoded by mtDNA, is necessary for growth under Al stress.

Regulation of TCA cycle and respiratory-chain enzymes, and ATP content. From the results described above, increased numbers of mitochondria and mtDNA copy number in resistant strains seemed to play an important role in Al resistance. Because mitochondria are the organelle where energy is produced, we measured enzyme activities involved in energy generation. From the results shown in Fig. 7, we conclude the following.

(i) In the presence of Al, wild-type cells downregulate two of the three NADH-producing steps in the TCA cycle (isocitrate dehydrogenase and malate dehydrogenase), which decreases the amount of NADH shunted to complex I. At the same time, complex I and IV, which generate membrane potential, were reduced by about 90%. Complex V, which reduces the membrane potential and produces ATP, was upregulated. These changes probably led to less NADH production, less mitochondrial membrane potential, and increased ATP production. Too high a mitochondrial membrane potential generally inhibits proton pump activity at complex IV, which leads to inhibition of overall electron transfer, where reduced or half-reduced ubiquinone accumulates as a potential source of superoxide radical (Brownlee, 2001; Jezek & Hlavata, 2005). Wild-type cells regulate the mitochondrial energy-generation system in order to lower membrane potential and lower ROS production under Al stress, resulting in adaptation to Al toxicity. Consistent with this, wild-type...
cells growing in the presence of Al generated negligible ROS (Fig. 3b).

(ii) On the other hand, regulation in the resistant cells was opposite to that observed in the wild-type cells, which was consistent with the morphological changes of the mitochondria (Fig. 2). This suggests that the resistant cells were highly adapted to avoid Al-induced stress such that they maintained cellular and mitochondrial homeostasis in the consistent presence of Al. Thus the smaller mitochondria with undeveloped cristae structures of resistant cells in the absence of Al appeared to be caused by their sudden adaptation to the new environmental change.

(iii) The activity of complex IV in resistant cells was reduced significantly to about 30% of wild-type levels. Complex IV uses oxygen to oxidize cytochrome c and produces a membrane potential. The reduction in activity represses electron transfer, thereby promoting ROS generation. The high ROS production in the resistant cells, even in the absence of Al (Fig. 3), might be caused by repression of complex IV.

(iv) Even though the resistant cells contained more mitochondrial mass than wild-type cells, the enzyme activities of the respiratory chain and TCA cycle were not much higher than in the wild-type cells. This suggests that the increased mitochondrial mass in the resistant cells was a compensatory response resulting from repression of essential mitochondrial activity. It has been reported that Al can substitute for iron (Fe) in Fe-dependent mitochondrial proteins (Middaugh et al., 2005). Energy-generating systems containing an Fe–S cluster, such as complexes I, II and III, are severely inhibited by Al. Thus, it is likely that cellular energy demand induces mitochondrial biogenesis under conditions of Al stress and Fe deprivation.

(v) Cellular ATP content increased in wild-type cells in the presence of Al, contrary to what was observed in resistant cells, which was consistent with complex V activity. As high ATP content has been reported in Al-tolerant cultivars of plants such as pea (Kobayashi et al., 2004) and tobacco cells (Yamamoto et al., 2002), maintenance of a high ATP content is possibly crucial for Al tolerance in wild-type
cells. On the other hand, we observed that the ATP content of resistant cells increased in the absence of Al. Together with the smaller mitochondria in the absence of Al, these results suggest that mitochondrial enzyme activity and resultant ATP content are concomitantly regulated with mitochondrial morphology changes.

Conclusions
The novel adaptive and heritable Al resistance found in R. glutinis was accompanied by several cellular and genetic changes, including changes in the numbers and sizes of mitochondria concomitant with Al-induced ROS production, changes in regulation of nuclear and mtDNA genes, and regulation of TCA-cycle and respiratory-chain activities. Changes in regulation of mitochondrial activity were found to be crucial for resistance, presumably through avoidance of Al-induced ROS-mediated damage. Maintenance of a minimal level of mitochondrial activity was found necessary for survival under Al stress. Wild-type cells were found to be tolerant to 50 μM Al through regulation of TCA cycle and respiratory-chain activities, while resistant cells were able to tolerate 1–5 mM Al by genetic adaptation, resulting in an increase in number of mitochondria and maintenance of mitochondrial activity. The regulation of nuclear-encoded genes found by DD analysis may possibly be involved in Al resistance, through direct or indirect interaction with mitochondria, which should be studied further.

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