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A Novel Radiation-Resistant Strain of *Filobasidium* sp. Isolated from the West Sea of Korea

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A novel radiation-resistant *Filobasidium* sp. yeast strain was isolated from seawater. Along with this strain, a total of 656 yeast isolates were purified from seawater samples collected from three locations in the West Sea of Korea and assessed for their radiation tolerance. Among these isolates, five were found to survive a 5 kGy radiation dose. The most radiation-resistant strain was classified as *Filobasidium* sp. based on 18S rDNA sequence analysis and hence was named *Filobasidium* RRY1 (Radiation-Resistant Yeast 1). RRY1 differed from *F. elegans*, which is closely related to RRY1, in terms of the optimal growth temperature and radiation resistance, and was resistant to high doses of γ-ionizing radiation (D_{10}: 6–7 kGy). When exposed to a high dose of 3 kGy irradiation, the RRY1 cells remained intact and undistorted, with negligible cell death. When these irradiated cells were allowed to recover, the cells fully repaired their genomic DNA within 3 h of growth recovery. This is the first report in which a radiation-resistant response has been investigated at the physiological, morphological, and molecular levels in a strain of *Filobasidium* sp.

Keywords: Ionizing radiation, radiation-resistant yeast, *Filobasidium* sp., *Saccharomyces cerevisiae*

Introduction

Ionizing radiation is an extreme stress that can severely damage a number of diverse cellular components, such as nucleic acids, proteins, and lipids, and can cause lethality to the majority of living organisms [22]. Nevertheless, there exist a small percentage of naturally ionizing radiation-resistant organisms [5, 19]. Ionizing radiation-resistant organisms have been isolated from a variety of different sources, including processed/canned food items, the paper industry, and soil and water samples [19]. These resistant organisms possess a highly efficient and robust system to survive exposure to high doses of ionizing radiation. The DNA and other cellular biomolecules of these organisms are severely damaged under ionizing radiation stress, but are able to recover and resume normal growth without any detectable mutations [22]. Among all radiation-resistant organisms, *Deinococcus* is the most studied bacterial model system. Strains of *Deinococcus* have been found to withstand g-ionizing radiation doses as high as 15 kGy [2, 5]. A dose of 5 kGy, which is quite lethal to other living organisms, only marginally affects the growth and survival of *Deinococcus* [2, 5]. The radiation-resistant phenotype in *Deinococcus* has been linked to various cellular defence mechanisms, such as an efficient and error-free DNA repair machinery, a multicopy genome, and the presence of a protective manganese-ion-dependent redox system [2, 5, 8, 9, 17]. *Deinococcus* is also resistant to other extreme stresses such as desiccation, suggesting an overlap between radiation stress and other environmental stresses [17].

Although radiation resistance has been extensively reported in bacteria and archaea, many fungal species, including *Aspergillus, Curvularia geniculata, Alternaria alternata, Cladosporium cladosporioides, Cryptococcus neoformans,* and

Ustilago maydis, have also been found to be resistant to ionizing radiation [7, 14, 21]. For instance, radiation-tolerant fungi have been isolated from the Chernobyl plant, where fungi colonized the walls under constant levels of high radiation [7, 11]. Fungi possess distinct morphological properties such as the presence of melanin and other pigments, which have been shown to contribute to this organism's robust radiotolerant phenotype [11]. Species of *Ustilago* and *Saccharomyces* are currently being used as model systems for studying radiation stress responses and adaptation mechanisms in fungi and yeast [3, 7, 14, 15]. Although current model systems have been thoroughly investigated to understand the mechanism of radiation stress tolerance and related stress responses, there is still a need to find new stress resistance systems, which will help further our understanding of the underlying molecular mechanisms behind these extreme stress responses. In support of this idea, different environmental sources have been continually explored for novel radiotolerant organisms. In the current study, we isolated a novel radiation-resistant yeast strain obtained from seawater, and investigated its radiation resistance.

Materials and Methods

Isolation Procedure

Seawater samples were collected from three locations in the West Sea of Korea: Maehwa Island, Chilsan Island, and Doripo Beach, located in Shin'an, Yeonggwang, and Muju, respectively, in Jeollanam Province. Seawater samples (50 ml) were filtered through 4.5 μm pore size cellulose nitrate filter papers and placed face-up on isolation medium plates containing 0.3% malt extract, 0.3% yeast extract, 0.35% NaCl, 0.5% peptone, 1% glucose, and 2% agar supplemented with 100 μg/ml chloramphenicol. The plates were incubated at 25°C for 14 days. After a single colony was isolated by successive streaking, the cells were stained with methylene blue to examine their shape. Only pure cultures with similar cell shapes were selected and transferred to malt extract agar slants for further studies.

Screening and Identification of Radiation-Resistant Yeasts

Selected isolates were cultivated at 25°C in YPD (1% yeast extract, 2% peptone, and 2% glucose) broth. Cultures grown for 7 days were spotted on YPD agar and subjected to 5 kGy of irradiation at room temperature (RT) using a ^{60}Cobalt γ-ray irradiator (point source, AECL, IR-79; MDS Nordion International Co. Ltd., Ottawa, Canada). The source strength was approximately 215 kCi at a dose rate of 10 kGy/h. The plates were then incubated at 25°C for 4 days for colony formation. The candidate with the highest radiation resistance was further used for strain identification using an 18S rDNA sequence analysis. Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, WI, USA), and 18S rDNA was PCR-amplified using universal primers (NS1, 5′-GTA GTC ATA TGC TTG TCT C-3′; and NS8, 5′-TCC GCA GGT TCA CCT ACG GA-3′) [23]. The amplicons were sequenced using an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems Inc., CA, USA) and then aligned and analyzed using the BLASTN program at the National Center for Biotechnology Information (NCBI) website.

Growth and Survival

Growth and survival of *Filobasidium* RRY1 was compared with that of *S. cerevisiae* SC7931 (Korean Culture for Type Collection, Korea) and *F. elegans* CBS7640 (CBS-KNAW Fungal Biodiversity Centre, The Netherlands). All strains were inoculated in YPD broth, and growth curves were studied until the stationary phase at 20°C and 25°C. For the survival studies, log phase cultures were exposed to different doses of γ-radiation, and dilutions were plated on YPD agar plates. Plates were incubated at 25°C for 4 days, and CFU (colony forming units) were counted.

Cellular Morphology and Membrane Integrity Assay

PI staining and FACS analysis. The positively charged fluorescent nucleic acid dye propidium iodide (PI; Molecular Probes) was used to stain the irradiated cells as follows [10]. Log phase cultures of *Filobasidium* RRY1 and *S. cerevisiae* SC7931 were exposed to 3 kGy of irradiation. Cells were washed and resuspended in phosphate-buffered saline (PBS, pH 7.4) and incubated with 10 μl of PI (50 μg/ml) for 10 min at RT. PI staining was followed by flow cytometry analysis to estimate cell viability [6, 10]. A Cytomics FC 500 flow cytometer (Beckman Coulter Inc., IN, USA) was used for single-cell light scattering and fluorescence measurements.

Scanning electron microscopy (SEM). *S. cerevisiae* SC7931 and *Filobasidium* RRY1 cells were exposed to 3 kGy of ionizing radiation and processed for SEM imaging. The cells were harvested by centrifugation and fixed with 2% (v/v) glutaraldehyde solution at RT for 4 h. The samples were washed with PBS and dehydrated using a graded ethanol series (30%, 50%, 70%, 80%, and 100%). The samples were then coated with gold–palladium and examined using a model JSM-30 scanning electron microscope (JEOL, Japan).

Fatty acid analysis. The cellular fatty acid (CFA) composition of the RRY1 strain was determined using a standard protocol of the MIDI/Hewlett Packard Microbial Identification System [1], in which fatty acid methyl esters of freeze-dried yeast cells (unirradiated or irradiated at 3 kGy) were extracted after saponification and methylation, and then analyzed by gas chromatography. The Agilent 6890N GC system (Agilent Technologies Inc., DE, USA) with a methyl phenyl silicone fused silica capillary column (HP 19092B-102) and flame ionization detector (FID) (carrier gas: H_2; initial temperature: 170°C; final temperature: 270°C; FID temperature: 300°C; injection port temperature: 250°C) was used for the analysis.

DNA Damage and Repair Kinetics

The extent of DNA damage and repair kinetics of *S. cerevisiae*

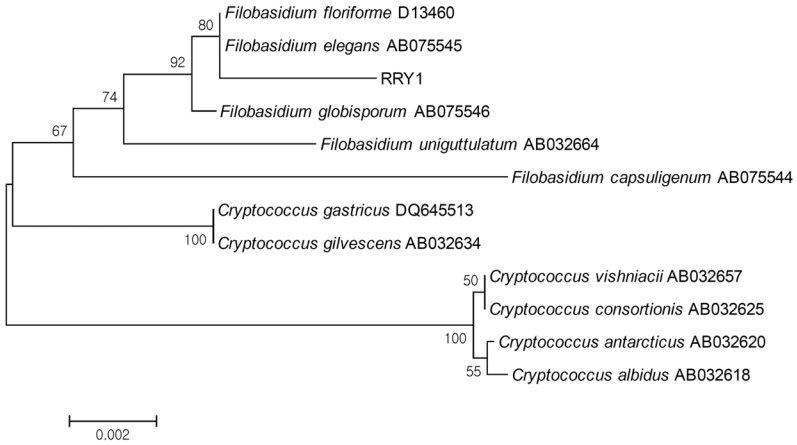

Fig. 1. Phylogenetic tree showing the evolutionary position of the RRY1 strain.
Phylogenetic tree based on 18S rDNA sequence data of the RRY1 strain. The tree was drawn using the neighbor-joining method [20]. Bar: 2 substitutions per 1,000 bases. The numbers at branching points are bootstrap values calculated from 1,000 replicates. Species names and their GenBank accession numbers are shown.

SC7931 and *Filobasidium* RRY1 was monitored using pulsed field gel electrophoresis (PFGE). Both *S. cerevisiae* SC7931 and *Filobasidium* RRY1 cells were grown to mid-log phase (OD_{600} = ~ 4.0) and exposed to γ-radiation (3 kGy). Post-irradiation, the cells were reinoculated into fresh YPD broth and incubated for growth recovery. At different post-irradiation recovery time points, culture aliquots (1 ml) were taken to prepare DNA plugs, as described in the manual of the CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad, Inc., US) with the following exception: lyticase was substituted with lysing enzymes (4 mg/ml) from *Trichoderma harzianum* (Sigma). The plugs were subjected to pulsed field gel electrophoresis for 24 h at 14°C using the CHEF Mapper XA System (Bio-Rad Laboratories, Inc., CA, USA) under the following conditions: 6 V/cm, linear pulse ramp of 60−120 sec, and a switching angle of 120° (−60° to +60°).

Results and Discussion

Isolation of Radiation-Resistant Yeast

A total of 656 yeast isolates were found from seawater from the West Sea of Korea. Among these, only five (named RRY1 through RRY5, radiation-resistant yeast) were highly resistant to ionizing radiation stress. RRY1 was the most radiation-resistant and was thus chosen for further identification and detailed investigations. The 18S rDNA sequence analysis showed that the RRY1 strain is closely related to *Filobasidium* according to phylogenetic analysis (Fig. 1). We compared the growth profile of our RRY1 strain with that of the *F. elegans* CBS7640 strain, which is one of two closely related strains (Fig. 1). The *F. elegans* strain showed a longer lag phase than that of RRY1 at 25°C (data not shown), while the growth profiles of the two strains were quite similar with a lag phase of approximately 10 h and a log phase spanning approximately 30 h at 20°C (Fig. 2A). To investigate the radiation resistance in these strains, both groups of cells were exposed to γ-radiation, spotted on YPD agar, and incubated at 20°C. At a 5 kGy dose, RRY1 cells demonstrated less than 1 log of cell death, whereas *F. elegans* (D_{10}: 1−2 kGy) cells showed approximately 5 logs of cell death (Fig. 2B). Taken together, these data indicate that RRY1 is more resistant to radiation than the closely related *Filobasidium* strain, *F. elegans*.

The radiation resistance of RRY1 and its comparison with *F. elegans* was demonstrated in the present study, but the radiation resistance phenotype in *Filobasidium* had not yet been reported earlier. Thus, to validate our findings, the radioresistant phenotype of RRY1 was compared with *Saccharomyces cerevisiae*, the widely known yeast model used for radiation resistance studies. RRY1 (D_{10}: 5−7 kGy) showed better growth and higher radiation resistance than *S. cerevisiae* SC7931 (D_{10}: <1 kGy) (Figs. 2C and 2D). In particular, at doses of 9 and 11 kGy, RRY1 was largely able to survive, demonstrating only 1- to 2-logs cell death, whereas *S. cerevisiae* SC7931 was completely inactivated by the same dose of γ-radiation (Fig. 2D). An earlier study reported the D_{50} dose for *S. cerevisiae* to be less than 1 kGy [3]. The present results show that the isolated strain RRY1 is significantly more radioresistant than *S. cerevisiae*.

F. elegans RRY1 Maintains Membrane Integrity after Irradiation

It is known that extreme ionizing radiation stresses target vital cellular macromolecules and membranes [12].

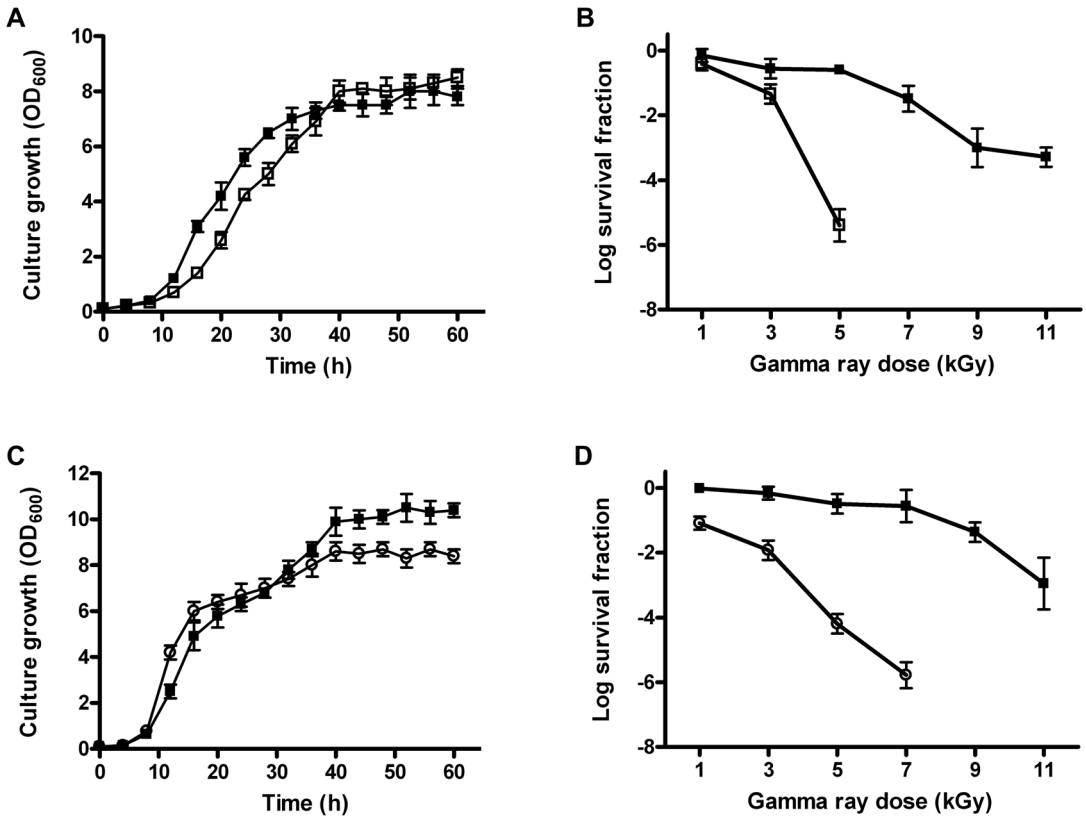

Fig. 2. Growth (**A** and **C**) and survival (**B** and **D**) of *Filobasidium* RRY1 [■], *F. elegans* CBS7640 [□], and *S. cerevisiae* SC7931 [○]. Cells were inoculated in YPD broth and their growth was monitored by measuring the absorbance at regular intervals up to the stationary phase (A and C). To examine survival, cells were exposed to different doses of γ-radiation, serially diluted in YPD medium, and plated on YPD agar plates (B and D). Cells were incubated at 20°C (A and B) and 25°C (C and D) for 4 days, after which colony forming units were calculated.

Highly reactive free radicals such as $OH^{\cdot}$ and $O_2^{\cdot-}$, generated by ionizing radiation, react with membrane lipids and proteins and result in lipid peroxidation [4, 16], protein damage, and changes in lipid bilayer polarity, which collectively affect the structure and function of cellular membranes [4]. The membrane permeability and integrity of irradiated RRY1 cells were assessed by staining the cells with a fluorescent stain PI [6, 10]. Cells with intact membranes are impermeable to PI and thus exclude the dye and remain non-fluorescent. Conversely, for cells with damaged membranes, PI can enter the cell, bind to the nucleic acids, and cause fluorescence [6, 10]. When irradiated *Filobasidium* RRY1 and *S. cerevisiae* cultures were incubated with the PI stain and analyzed through flow cytometry, the cells segregated into two types of cell populations: viable unstained cells with intact membranes and stained cells with damaged membranes (Fig. 3). Following exposure to 3 kGy of γ-radiation, *S. cerevisiae* SC7931 cells showed a drastic increase in the percentage of cells (62.5%) with damaged membranes and permeability to PI. Conversely, a very small percentage of cells (8.7%) from the *Filobasidium* RRY1 strain showed permeability to PI under the same conditions (Fig. 3).

Damage to cellular membranes under ionizing radiation was shown by PI staining. To further confirm the phenotype, irradiated and unirradiated cells were also observed by SEM. Following exposure to 3 kGy of γ-irradiation, *S. cerevisiae* SC7931 cells had a highly distorted cellular morphology, likely owing to a disrupted membrane integrity and intracellular osmotic balance; whereas *Filobasidium* RRY1 cells were intact and morphologically similar to unirradiated control cells (Fig. 4). Cellular membrane integrity was also assessed by monitoring the fatty acid composition post-irradiation. Compared with unirradiated cells, fatty acids fragmentation was clearly evident in the irradiated *S. cerevisiae* SC7931cells, whereas it was marginally changed in the irradiated *Filobasidium* RRY1 cells (Table 1). Additionally, the percentage of palmitic acid (C16) was

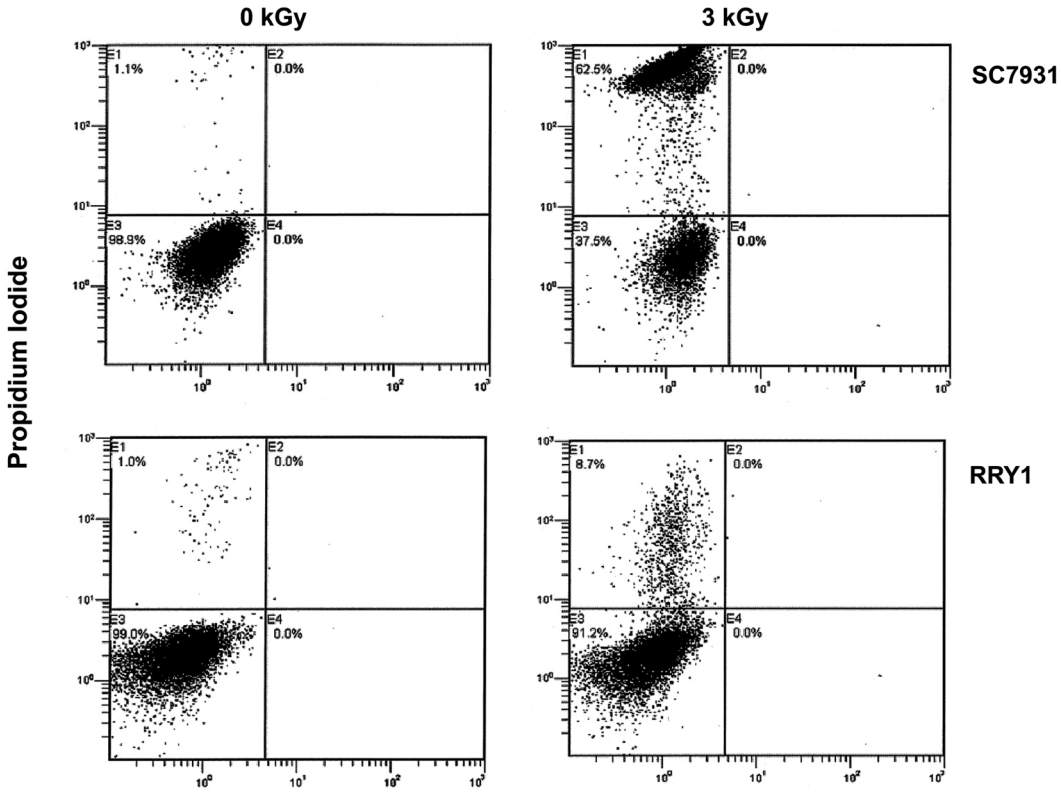

Fig. 3. Effect of 3 kGy γ-radiation on cell viability.
Cells of *S. cerevisiae* SC7931 and *Filobasidium* RRY1 were exposed to 3 kGy of ionizing radiation. Unirradiated (0 kGy) or irradiated (3 kGy) cells of *S. cerevisiae* SC7931 and *Filobasidium* RRY1 were stained with propidium iodide, followed by flow cytometry analysis.

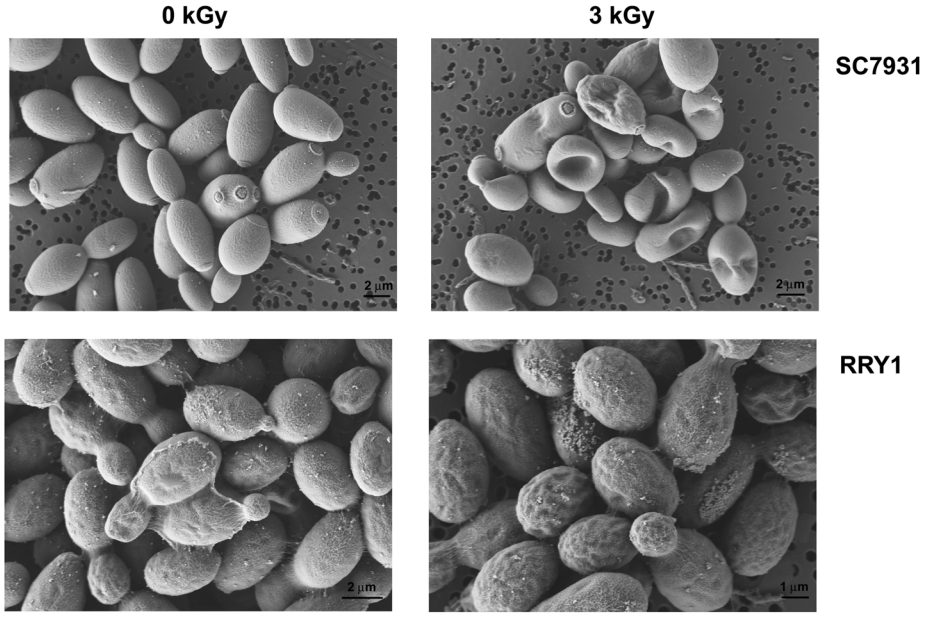

Fig. 4. Post-irradiation cell morphology.
Cells of *S. cerevisiae* SC7931 and *Filobasidium* RRY1 were exposed to 3 kGy of γ-ionizing radiation and immediately processed for scanning electron microscopy.

Table 1. Changes in fatty acid composition following irradiation.

Fatty acid	*S. cerevisiae*		Fatty acid	*Filobasidium* RRY1	
	0 kGy (%)	3 kGy (%)		0 kGy (%)	3 kGy (%)
C10:0	ND[a]	5.58 ± 0.88			
C12:0	ND[a]	2.82 ± 0.40			
C14:0	ND[a]	2.21 ± 0.06			
C16:1 *cis*-9 (w7)	53.85 ± 0.78	44.79 ± 1.95	C18:2 *cis*-9,12/18:0a	45.73 ± 0.81	45.81 ± 0.79
C16:0	9.08 ± 0.40	15.49 ± 0.23	C16:0	14.46 ± 0.21	14.17 ± 0.11
SIF 8[b]	34.79 ± 1.15	27.69 ± 1.03	SIF 8[b]	39.10 ± 0.01	37.23 ± 0.26
C18:0	2.29 ± 0.04	2.88 ± 0.58	C18:0	1.44 ± 0.65	2.80 ± 0.42

[a]ND, not detected.

[b]Summed in feature (SIF) 8, C18:1 *cis*-9 (w9) and/or C18:1 (w8).

considerably increased in the *S. cerevisiae* SC7931 cells after exposure to 3 kGy of irradiation (Table 1). Because an increase in C16 results in membrane reconstitution and a subsequent loss in cell viability, this finding indicates the membrane damage and cell death of these cells [18].

RRY1 Cells Can Rapidly Reconstitute Their Genomic DNA After Irradiation.

It is well known that radiation-resistant organisms are equipped with versatile and efficient DNA repair systems [2]. To assess the DNA damage repair capability of RRY1, the kinetics of DNA repair in *Filobasidium* RRY1 was investigated and compared with that of *S. cerevisiae* SC7931 during post-irradiation recovery. The unirradiated culture samples showed 14 distinct chromosomal DNA bands, whereas in the irradiated samples, the chromosomal DNA profile was missing, likely owing to severe DNA damage upon 3 kGy of irradiation (Fig. 5). During post-irradiation recovery, the normal chromosomal DNA profile was restored in the *Filobasidium* RRY1 strain after 3 h of growth. In contrast, the *S. cerevisiae* SC7931 strain was not able to repair its severely damaged DNA, and the chromosomal DNA profile was not restored, even after 4 h of growth (Fig. 5). Together, these data indicate that the RRY1 strain is equipped with superior DNA repair capability during post-irradiation recovery and thereby has remarkable radiation resistance.

In summary, a novel radioresistant strain, *Filobasidium* sp. RRY1, was found in seawater from the West Sea of Korea. RRY1 showed robust radioresistant capacity (D_{10}: 6–7 kGy), which was directly comparable to that of the extreme radiation-resistant bacteria *Deinococcus radiodurans* R1 (D_{10}: 10–15 kGy) [2, 5, 19] and more robust than that of the model radioresistant fungus *Ustilago maydis* (D_{37}: 3.6–6 kGy) [14]. There have been reports on the radiation resistance of *Cryptococcus*, particularly of the widely known pathogenic strain *C. neoformans* [7]; however, these organisms have been grouped under the Filobasidiella group [13]. Phylogenetic studies have shown that these organisms are clearly separate and distant from the *Filobasidium* group [13]. It is quite evident from our results that *Filobasidium* RRY1 is highly radioresistant, which is achieved by

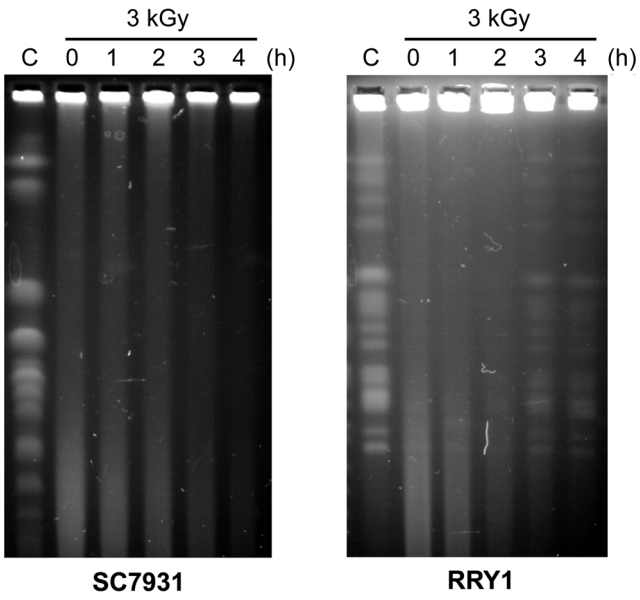

Fig. 5. DNA damage and repair kinetics.
Exponential phase *S. cerevisiae* SC7931 and *Filobasidium* RRY1 cells were exposed to 3 kGy of γ-irradiation, reinoculated into fresh YPD broth, and incubated for growth recovery. Samples were collected at different post-irradiation recovery time points and processed as described in the manual of CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad) and subjected to pulsed field gel electrophoresis at 6 V/cm for 24 h at 14°C using a CHEF Mapper XA System (Bio-Rad), with pulse ramp from 60–120 sec.

maintaining membrane integrity after exposure to high doses of ionizing radiation. RRY1 cells were able to repair severely damaged DNA as efficiently as radioresistant *Deinococcus radiodurans* [2, 5, 17] and *Ustilago maydis* [15]. The discovery of a new eukaryotic strain with robust radiation resistance will provide further insight into the underlying complex mechanism of extreme stress tolerance such as radiation resistance in living organisms.

Acknowledgments

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