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Suppressive Effect of Wild *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* Strains on Ige Production by Mouse Spleen Cells

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The genus *Saccharomyces* includes industrial yeasts that are used for bread and alcoholic beverage production. *Saccharomyces* strains isolated from natural resources, referred to as "wild" yeasts, are used for making products with strain-specific flavors that are different from those of the "domesticated" industrial yeasts. The physiological effects of wild yeast are poorly understood. In this study, we isolated 2 *Saccharomyces cerevisiae* strains (S02 − 03) and 5 *Saccharomyces paradoxus* strains (P01 − 02, S01, S04 − 05) from natural resources in the *Kiso* area and investigated the effect of these fungal strains on IgE production by mouse spleen cells. Culturing spleen cells with heat-killed yeasts resulted in elevated IFN-γ and IL-12 levels followed by significant reduction in IgE levels. The S03 and P01 strains induced IL-12 p40 and IL-10 expression in RAW264 cells. Thus, wild strains of *S. cerevisiae* and *S. paradoxus* regulate macrophage cytokine production to improve the Th1/Th2 immune balance and suppress IgE production.

Keywords: *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, wild yeast, IFN-γ, IL-12, IgE, allergy

Introduction

The yeasts belonging to the genus *Saccharomyces sensu stricto* have long been used for fermented food and alcoholic beverage production because of their metabolic properties that enable conversion of sugar into ethanol and CO_2 (Rainieri *et al.*, 2003; Sicard and Legras, 2011). In particular, *Saccharomyces cerevisiae*, *S. bayanus*, and *S. pastorianus* (*S. carlsbergensis*) have been empirically considered non-pathogenic to humans, and are commonly used as starter cultures (brewer's yeast, baker's yeast, and wine yeast, respectively) for food and beverage production on an industrial scale. Moreover, *Saccharomyces* yeasts are consumed as nutritional supplements.

On the other hand, non-industrial yeast strains have been isolated from many natural resources such as fruits, tree sap, flowers, and soil. These yeasts are frequently referred to as "wild" yeasts in contradistinction to "domesticated" industrial yeasts and have been extensively used for alcoholic fermentation. Some wild *Saccharomyces* yeasts produce molecules with unique sensory flavors, which are not obtained from common industrial yeast. To date, some of the favorably fermented foods obtained by using wild *Saccharomyces* strains have been introduced into the market and have become popular (Oda *et al.*, 2010).

Despite their contribution in sensory function, physiological functions provided by *Saccharomyces* yeasts have been reported only for a single special strain. *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii*) has been extensively studied for its role in attenuating the toxicity of several enteric bacterial pathogens (Gedak, 1999; Wu *et al.*, 2008), maintaining epithelial barrier integrity (Dahan *et al.*, 2003; Klingberg *et al.*, 2008), treating and preventing gastrointestinal diseases (Zanello *et al.*, 2009), and reducing inflammatory responses triggered by bacterial infection (Lee *et al.*, 2005). The probiotic properties of *S. boulardii* have been determined by double-blind, randomized, and placebo-controlled studies (Bleichner *et al.*, 1997; Sazawal *et al.*, 2006). Notably, *S. boulardii* is the only probiotic yeast strain that is utilized as a functional food ingredient today.

S. boulardii stimulates intestinal immunity by enhancing immunoglobulin (Ig)A production. Buts *et al.* (1990) reported that orally administered *S. boulardii* induced mucosal secretory IgA in the small intestine of rat. In addition,

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Lessard *et al.* (2009) reported that the intake of *S. boulardii* modulated the development of intestinal IgA secretion and reduced intestinal bacterial translocation in pigs. Thus, these reports imply that orally administered *Saccharomyces* yeasts have the potential to regulate Ig production. However, little is known about the effect of *Saccharomyces* strains, including various wild strains, on the production of other classes of Igs.

In this study, we isolated 2 *S. cerevisiae* and 5 *S. paradoxus* strains from fruit and soil samples and investigated the effect of those strains on IgE production, an Ig class closely associated with the pathophysiology of type-I allergy (Gould *et al.*, 2003). Among the isolated *Saccharomyces* strains and type strains of the same species, *S. cerevisiae* S03 and *S. paradoxus* P01 showed the strongest IgE suppressive effect. The IgE suppressive effect of these strains was thermostable at 200°C for 40 min. These strains significantly increased interferon (IFN)-γ and interleukin (IL)-12 production in spleen cells and increased IL-12 p40 and IL-10 mRNA expression in macrophage-like RAW264 cells. Thus, some wild strains of *S. cerevisiae* and *S. paradoxus* can be used as IgE-suppressive agents in functional foods.

Materials and Methods

Materials The isolation medium for *Saccharomyces* yeasts was prepared as follows: Approximately, 150 g of commercially available dried rice malt (Kojiya Mitsuemon, Aichi, Japan) was immersed in 450 mL of distilled water and incubated at 56°C for 6 h for saccharification. The saccharified liquid was then centrifuged at 3000 × *g* for 10 min. The supernatant was sterilized using a 0.2-μm pore cellulose acetate membrane filter (Advantec, Tokyo, Japan). The filtrate was supplemented with 2% lactic acid, 0.2% sodium propionate, and 0.01% chloramphenicol for use as isolation medium (pH 3.1).

Type strains of *S. cerevisiae* (NBRC 10217) and *S. paradoxus* (NBRC 10609) were purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC; Chiba, Japan). These strains were cultured in yeast-peptone-dextrose (YPD) broth (1% yeast extract, 2% peptone, and 2% D-glucose; pH 5.8) and stored as frozen cultures in the medium at −80°C.

Mouse leukemic monocyte cell line RAW264 was obtained from Riken Cell Bank (Ibaraki, Japan) and maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA).

Isolation and cultivation of yeast strains For yeast isolation, 208 sample resources, including fruits, flowers, leaves and soil, were collected from the *Kiso* area (Nagano, Japan). The enrichment culture method was used to isolate *Saccharomyces* yeasts as follows: Sample resources were submerged in 10 − 50 mL of isolation medium and incubated at 29°C for 1 week, following which, 1 mL of the forming culture supernatant was mixed with 13 mL of yeast-peptone-sucrose (YPS) broth (1% yeast extract, 2% peptone, and 2% sucrose; pH 5.8) supplemented with 3% ethanol, 2% lactic acid, 0.2% sodium propionate, and 0.01% chloramphenicol and incubated at 29°C under anaerobic conditions. After 10 days, 1 mL of the forming culture supernatant was seeded in a YPD agar plate (pH 6.8) containing 1.5% (w/v) agar and incubated at 29°C for 2 days. The yeast-like 4970 colonies were then transferred to fresh YPD broth. The yeast strains grown in the YPD broth at 29°C were stored at −80°C until use.

Classification of yeast strains Isolated yeast-like strains were provisionally classified using the API ID32 C identification kit (Sysmex-bioMérieux; Marcy l'Etoile, France) according to the manufacturer's protocols.

Among the 16 strains classified as *S. cerevisiae* in the API ID32 C test, seven strains that suppressed IgE production were further evaluated by comparing the partial D1/D2 26S rDNA sequences with the DNA sequences provided by Techno Suruga (Shizuoka, Japan) using the National Center for Biotechnology Information (NCBI) database and the standard nucleotide-nucleotide homology search tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Preparation of heat-killed yeasts Isolated *Saccharomyces* strains were cultured in YPD medium at 30°C for 24 h and then harvested by centrifugation at 1000 × *g* for 10 min. The yeast pellet was washed 3 times with distilled water and then lyophilized.

The lyophilized yeasts were heated at 70°C for 30 min in a thermal cycler (PTC-200; MJ Research, Waltham, MA) and used as heat-killed yeasts. For evaluating the effect of baking temperatures on the bioactivity of yeasts, the lyophilized yeasts were incubated at 200°C for 0, 20, 30, or 40 min prior to the heat inactivation.

Mice Specific pathogen-free male BALB/c mice aged 6 weeks were purchased from Japan SLC (Shizuoka, Japan) and housed at 23°C ± 3°C under a 12-h light/dark cycle. All the animal protocols used in this study were approved by the Committee for Animal Experiments of Shinshu University.

Mouse spleen cell culture Spleen cells were prepared from 6 to 8-wk-old mice according to a previously described method (Kawahara *et al.*, 2006). Briefly, mice were euthanized using the vertebral-dislocation method, and the spleens were aseptically harvested. A single-cell suspension was prepared by gentle manipulation of the spleen in RPMI 1640 medium. To remove the red blood cells, the spleen cells were treated with hemolytic buffer (17 mM Tris-HCl buffer containing 0.144 M ammonium chloride; pH 7.2) for 5 min

at room temperature, then diluted with fresh RPMI 1640 medium (same volume as the hemolytic buffer), and centrifuged at 450 × *g* for 10 min to remove the hemolytic buffer. The pelleted cells were resuspended in RPMI 1640 medium containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Enzyme-linked immunosorbent assay (ELISA) Spleen cells were seeded at a density of 5×10^6 viable cells/mL in Falcon 96-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA). The spleen cells were cultured with indicated concentrations of each heat-killed yeast strain, *S. cerevisiae*-derived zymosan (Sigma Aldrich, St. Louis, MO, USA), or *S. cerevisiae*-derived mannan (Sigma) at 37°C in a humidified atmosphere of 5% CO_2/95% air for 7 days to measure IgE production, or for 3 days to measure IFN-γ and IL-12 production. After cultivation, culture supernatant was collected. In the case of the evaluation of IgE production, mice were sensitized with ovalbumin (OVA) absorbed on Alum LG-6000 (Cosmo Bio, Tokyo, Japan) by intraperitoneal injection prior to the collection of spleen cells according to the method by Segawa *et al.* (2008). All culture supernatants for ELISA were stored at −80°C until use.

Levels of total IgE, IL-12, and IFN-γ in the spleen culture supernatants were measured by sandwich ELISA. Fifty microliters of the first antibodies dissolved in 0.1 M carbonate buffer (pH 10.0) were added to each well of a 96-well Nunc Immuno Plate MaxiSorp (Thermo Fisher Scientific, Roskilde, Denmark) and incubated at 37°C for 90 min. The concentration of the first antibodies for IgE, IL-12 p40/70, and IFN-γ measurement was as follows: 10 μg/mL goat anti-mouse IgE (Bethyl Laboratories, Montgomery, AL), 10 μg/mL rat anti-mouse IL-12 p40/70 (BD Pharmingen, San Diego, CA, USA), and rat anti-mouse IFN-γ (BD Pharmingen). Each well was washed three times with PBS (pH 7.2) containing 0.05% Tween 20 (TPBS), and then post-coated with 300 μL of Block Ace (DS Pharma Biomedical, Osaka, Japan) in 0.1 M sodium carbonate buffer (pH 10.0) at 4°C overnight. After the plates were washed three times with TPBS, 50 μL of the culture supernatant or standard solution at an optimal dilution with PBS was added to each well and was incubated at 37°C for 60 min. For measurement of IgE, IL-12, and IFN-γ, mouse IgE (Sigma), recombinant murine IL-12 (PeproTech, Rocky Hill, NJ, USA) or recombinant murine IFN-γ (PeproTech) was used as standard, respectively. The plates were then washed five times with TPBS, placed in 100 μL of second antibody dissolved in PBS, and incubated at 25°C for 60 min. The concentration of the second antibodies for measurement of IgE, IL-12, and IFN-γ was as follows: 2 μg/mL goat anti-mouse IgE horseradish peroxidase (HRP)-conjugated (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), 2 μg/mL rat anti-mouse IL-12 p40/70 biotin-conjugated (BD Pharmingen) and rat anti-mouse IFN-γ biotin-conjugated (BD Pharmingen). In the case of the measurement for IL-12 and IFN-γ, after washing five times with TPBS, the wells were filled with 100 μL of 2 μg/mL HRP-conjugated streptavidin (BD Pharmingen) and incubated at 25°C for 60 min. After washing five times with TPBS, 100 μL of TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry) was added to each well, and then incubated at 25°C for 15 min. The reaction was stopped by adding 100 μL of 1 M phosphoric acid. Absorbance at 450 nm was read on an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Preparation of total RNA and reverse transcription-polymerase chain reaction (RT-PCR) RAW264 cells (2×10^6 cells) were cultured with indicated concentrations of each heat-killed yeast strain, zymosan, or mannan for 6 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted RNA (1 μg) was reverse transcribed in PTC-200 with 1 mM each dNTP, 2.5 units/μL M-MLV reverse transcriptase (Invitrogen), and 10 pmol/μL of oligo$(dT)_{18}$ primers at 42°C for 50 min. The resulting cDNA was subjected to semiquantitative PCR.

PCR using 1 μg of cDNA was performed using a RBC *Taq* DNA Polymerase (RBC Bioscience, Taipei, Taiwan) and 10 pmol/μL primers. The primer sequences for IL-10 were designed as 5′-AGCAGCCTTGCAGAAAAGAG-3′ (forward) and 5′-AGGGTCTTCAGCTTCTCACC-3′ (reverse) from bases 39 − 58 and 423 − 404, respectively, from GenBank accession number NM_010548.2. The primer sequences for IL-12 p40 were designed as 5′-TTTTGCTGGTGTCTCCACTC-3′ (forward) and 5′-AACCGTCCGGAGTAATTTGG-3′ (reverse) from bases 203 − 222 and 572 − 553, respectively, from GenBank accession number NM_008352.2. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed as 5′-CATCTTCCAGGAGCGAGACC-3′ (forward) and 5′-AGTGATGGCATGGACTGTGG-3′ (reverse) from bases 269 − 288 and 590 − 571, respectively, from GenBank accession number NM_008084.2. PCRs were run for 30 cycles of denaturation (94°C, 1 min), primer annealing (60°C, 1 min), and extension (72°C, 1 min) using the PTC-200. Amplified cDNAs were electrophoresed on 2% agarose gels in 0.04 M tris-acetate buffer (pH 8.0) containing 1 mM EDTA, and visualized by ethidium bromide staining. The fluorescent intensities of the bands were digitized with E-graph AE-9000 (Atto, Tokyo, Japan) and ImageSaver5 software (Atto).

Statistical analysis Data were statistically analyzed us-

ing a two-sided Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

Results

Characteristics of isolated yeast strains Seven yeast strains (P01, P02, and S01 − 05) were isolated by enrichment culture and were classified using the API ID32 C test as *S. cerevisiae* with 99.7 − 99.9% accuracy (Table 1). Each of the 7 isolates showed fermentation patterns that included the assimilation of D-glucose, D-galactose, sucrose, maltose, raffinose, lactic acid, methyl-α-D-glycoside, palatinose, and D-melicitose. NBRC 10217 strain showed fermentation patterns including D-glucose, D-galactose, sucrose, lactic acid, raffinose, maltose, and palatinose. All strains form opaque white domed colonies on yeast mold (YM) agar plates. Scanning electron microscope analysis of the 7 strains revealed multipolar budding and ascospore formation in all strains (data not shown). A gene sequence comparison of the D1/D2 rDNA region identified 2 strains (S02 and S03) as *S. cerevisiae* NBRC 10217 (NRRLY-12632; accession number U44806). In addition, gene sequence comparison identified 5 strains (P01, P02, S01, S04, and S05) as *S. paradoxus* NBRC 10609 (NRRLY-17217; accession number U68555).

Effect of isolated yeast strain on IgE production in mouse spleen cells Spleen cells obtained from BALB/c mice immunized with OVA and Alum LG-6000 were incubated with the 7 isolated *Saccharomyces* strains and *S. cerevisiae* and *S. paradoxus* type strains. As shown in Fig. 1, all strains of heat-killed *S. cerevisiae* and *S. paradoxus* suppressed IgE production. The strongest suppressive effect was exerted by *S. cerevisiae* S03 strain and *S. paradoxus* P01, P02, and S01 strains. In contrast, both *S. cerevisiae* NBRC 10217 and *S. paradoxus* NBRC 10609 had negligible effects on IgE production. *S. cerevisiae* strains (S02 and S03) and *S. paradoxus* strains (P01, P02, and S01) induced a greater decrease ($p < 0.05$) in IgE production than that induced by NBRC 10217 and NBRC 10609, respectively.

Heat-stability of the IgE suppressive effect of S03 and P01 strains in mouse spleen cells To evaluate the thermostability of the suppressive effect on IgE production mediated by isolated yeast at temperatures used for bread baking, the *S. cerevisiae* S03 and *S. paradoxus* P01 strains were heated at 200°C for 0, 20, 30, or 40 min, followed by heat inactivation by incubation at 70°C for 30 min. As shown in Fig. 2, the suppressive effect of S03 strain on IgE production was not reduced by the high-temperature heating and seemed to strengthen with increasing heating time. Although the effect of P01 strain seemed to be weakened by high-temperature heating for 30 min or longer, significant suppression was retained compared to control.

Effect of S03 strain, P01 strain, and yeast components on IgE production in mouse spleen cells The effect of various concentrations of S03 strain, P01 strain, and commercially available *S. cerevisiae*-derived yeast components on IgE pro-

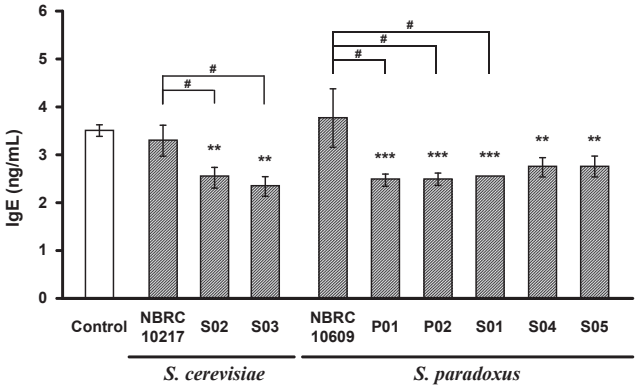

Fig. 1. Effect of isolated *S. cerevisiae* and *S. paradoxus* strains on IgE production in mouse spleen cells.

Spleen cells were cultured with 100 μg/mL of each yeast strain. The result shown is representative of three independent experiments. Data are expressed as mean ± standard deviation (SD) (n = 3). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. Control (unstimulated cells). $^{\#}P < 0.05$ vs. the respective type strain-stimulated cells.

Table 1. Isolated *Saccharomyces* strains and their fermentation patterns of carbon resources in API ID 32C test

Strain name	Source of isolation	Utilization									Identification
		GLU	GAL	SAC	MAL	RAF	LAT	MDG	PLE	MLZ	
P01	Hardy kiwifruit	○	○	○	○	○	△	○	○	△	*S. cereviasiae* (99.9%)
P02	Mulberry	○	○	○		○		○	○		*S. cereviasiae* (99.7%)
S01	Soil	○	○	○		○		○	○		*S. cereviasiae* (99.7%)
S02	Soil	○	○	○		○		○	○		*S. cereviasiae* (99.7%)
S03	Soil	○	○	○		○		○	○		*S. cereviasiae* (99.7%)
S04	Soil	○	○	○	○	○	○	○	○	○	*S. cereviasiae* (99.9%)
S05	Soil	○	○	○	○	○	△	○	○	○	*S. cereviasiae* (99.7%)
NBRC 10217	(Brewer's top yeast)	○	○	○	○	△			△		*S. cereviasiae* (99.7%)

GLU, D-glucose; GAL, D-galactose; SAC, saccharose (sucrose); MAL, maltose; RAF, raffinose; LAT, lactic acid; MDG, methyl-α-D-glucoside; PLE, palatinose; MLZ, D-melicitose.

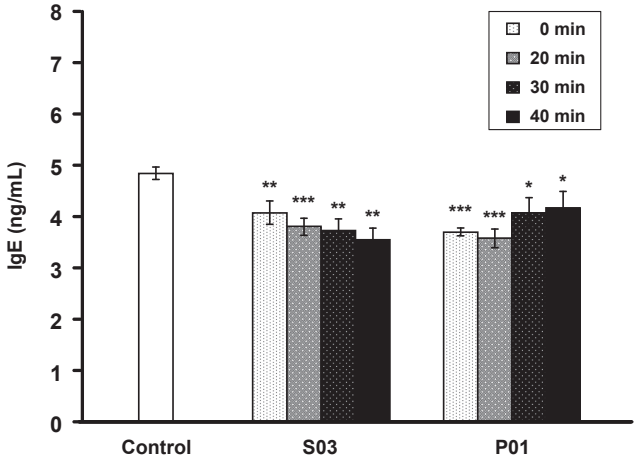

Fig. 2. Heat-stability of the IgE suppressive effect of S03 and P01 strains in mouse spleen cells.

Spleen cells were cultured with 100 μg/mL of S03 and P01 strains. The result shown is representative of two independent experiments. Data are expressed as mean ± SD (n = 3). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. Control (unstimulated cells).

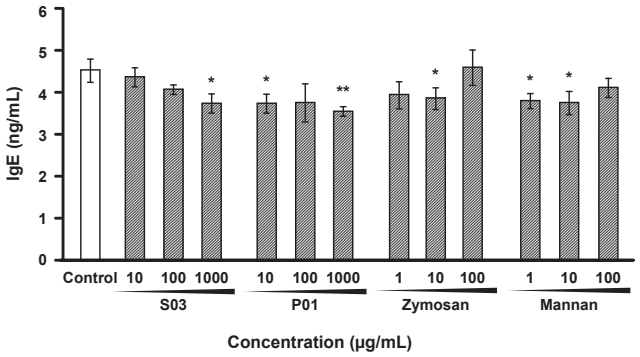

Fig. 3. Effect of S03 strain, P01 strain, and yeast components on IgE production in mouse spleen cells.

Spleen cells were cultured with 10, 100 and 1,000 μg/mL of S03 and P01 strains and 1, 10, 100 μg/mL of zymosan and mannan. The result shown is representative of two independent experiments. Data are expressed as mean ± SD (n = 3). *$P < 0.05$ and **$P < 0.01$ vs. Control (unstimulated cells).

duction was evaluated (Fig. 3). Culturing with 1000 μg/mL S03 strain and with 1 and 1000 μg/mL P01 strain resulted in significant suppression of IgE production from mouse spleen cells. Zymosan at 10 μg/mL and mannan at 1 and 10 μg/mL suppressed IgE production at concentrations of 10 μg/mL; however, neither zymosan nor mannan suppressed IgE production at 100 μg/mL.

Effect of S03 strain, P01 strain, and yeast components on IFN-γ and IL-12 production in mouse spleen cells The effect of S03 strain, P01 strain, and yeast components on IFN-γ and IL-12 production in mouse spleen cells is shown in Fig. 4A and Fig. 4B, respectively. The IFN-γ production was significantly higher in spleen cells stimulated by S03 strain (100 and 1,000 μg/mL) or P01 strains (10, 100, and 1000 μg/mL) compared with unstimulated control (Fig. 4A). Zymosan also significantly enhanced IFN-γ expression at concentrations of 10 and 100 μg/mL.

IL-12 production in spleen cells was enhanced by S03 and P01 strains, effects that peaked at 100 μg/mL (Fig. 4B). Comparable level of the peak enhancement was shown by 10 μg/mL zymosan. Stimulation with 100 μg/mL zymosan showed notably strong enhancement of IL-12 production. In contrast, mannan had no inductive effect on IL-12 production at all tested concentrations.

Effect of S03 strain, P01 strain, and yeast components on IL-12 p40 and IL-10 mRNA expression in RAW264 cells The effect of S03 strain, P01 strain, and yeast components on the expression for IL-12 p40 and IL-10 in RAW264 cells is shown in Fig. 5. The S03 and P01 strains at the concentrations of 100 μg/mL and 1000 μg/mL induced IL-10 and IL-12 p40 expression. Zymosan (100 μg/mL) strongly induced

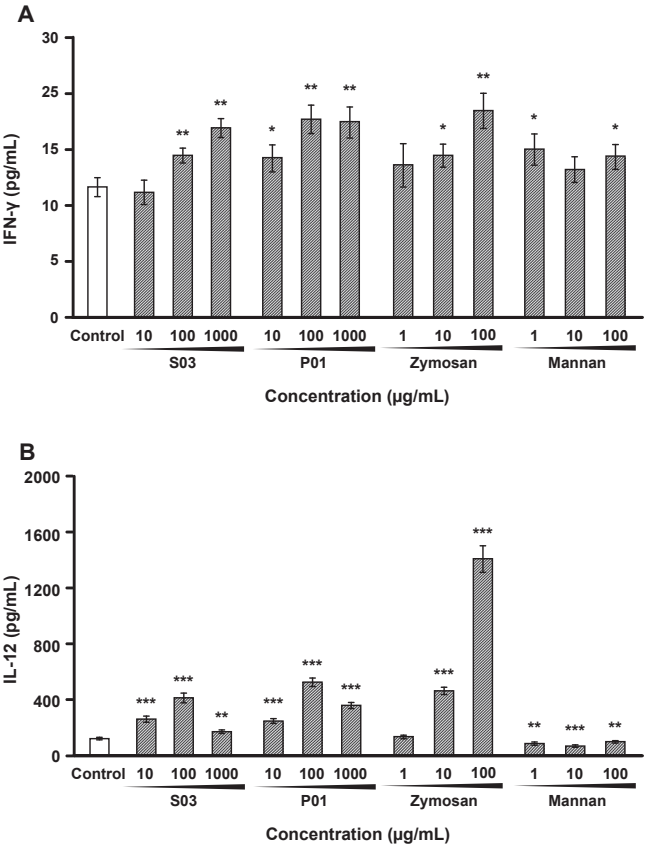

Fig. 4. Effect of S03 strain, P01 strain, and yeast components on cytokine production in mouse spleen cells.

A, IFN-γ; B, IL-12. Spleen cells were cultured with 10, 100 and 1,000 μg/mL of S03 and P01 strains and 1, 10, and 100 μg/mL of zymosan and mannan. The result shown is representative of two independent experiments. Data are expressed as mean ± SD (n = 3). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. Control (unstimulated cells).

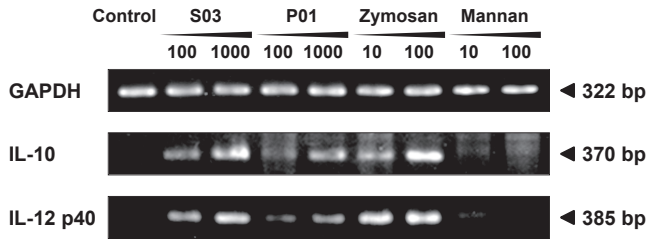

Fig. 5. Effect of S03 strain, P01 strain, and yeast components on cytokine expression in RAW264 cells.

RAW264 cells were cultured with 100 and 1,000 μg/mL of S03 and P01 strains and 10 and 100 μg/mL of zymosan and mannan. The result shown is representative of two independent experiments.

IL-10 and IL-12 expression. Mannan had a negligible effect on the expression of both cytokines.

Discussion

The probiotic properties of yeast species and strains, other than *S. boulardii*, need to be investigated (Moslehi-Jenabian *et al.*, 2010). In this study, we investigated the effect of wild *Saccharomyces* strains on IgE production.

A recent population genomics study evaluated differences in the genomic and phenotypic variation of wild *Saccharomyces* yeasts (Liti *et al.*, 2009). As shown in Table 1, carbon resource fermentation patterns of all isolated yeast strains clearly differed from that of strain NBRC 10217. Further gene analysis of the partial D1/D2 rDNA sequences of the 7 strains revealed that 2 strains (S02 and S03) were *S. cerevisiae* and 5 strains (P01, P02, S01, S04, and S05) were *S. paradoxus*. *S. paradoxus* was first reported in 1914 (Bachinskaya, 1914) and has been classified as a separate species distinct from *S. cerevisiae* since 1998 (Kurtzman and Fell, 1998). Currently *S. paradoxus* is the closest known species relative of *S. cerevisiae*, a representative *Saccharomyces* species affirmed as GRAS (generally recognized as safe; Goddard and Burt, 1999), and potentially edible. In fact, recent reports show that the quality of wine can be partially attributed to the production of fermentation-related aromatic compounds by *S. paradoxus* (Majdak *et al.*, 2002; Orlic *et al.*, 2007).

Fig. 1 shows that 2 *S. cerevisiae* and 3 *S. paradoxus* strains showed significant suppressive effect on IgE production as compared with the type strains, which had little effect on IgE production under the same experimental conditions. These results suggest that *S. cerevisiae* and *S. paradoxus* yeast strains, especially 5 strains, have a unique suppressive effect on IgE production.

We further investigated the thermostability of the suppressive effect by using high temperatures comparable to those used in the common bread baking process. The S03 and P01 strains, which exhibited the strongest suppressive activity among *S. cerevisiae* and *S. paradoxus* strains, were able to suppress IgE production when heated at 200°C for up to 40 min (Fig. 2). These results suggest that the suppressive effect of these strains on IgE production can be retained during the common entire bread-baking process.

As shown in Fig. 3, S03 and P01 strains, as well as yeast cell wall components zymosan and mannan, suppress IgE production from spleen cells. Thus, the suppressive effect of S02 and P01 strains on IgE production could be partially attributed to cell wall components. Many functional studies of laboratory strains of *S. cerevisiae* have demonstrated that the 2 carbohydrate cell wall components, β-glucan and mannan, are involved in the recognition by immune cells (Giaimis *et al.*, 1993). The main component of β-glucan of *S. cerevisiae* is a branched β-(1,3)-linked glucose of high molecular weight with β-(1,6)-glucosidic interchain linkages (Manners *et al.*, 1973a, Manners *et al.*,1973b). Zymosan consists of β-glucan-containing structures forming a protein-carbohydrate complex (Di Carlo and Fiore, 1958), and is a widely used immunomodulatory agent that is recognized by both Toll-like receptors 2/6 and CD14 in cooperation with dendritic cell-associated lectin-1 (Dectin-1) (Ozinsky *et al.*, 2000; Gantner *et al.*, 2003). The mannan in *S. cerevisiae* is linked by a series of oligosaccharides containing α-(1,2)-, α-(1,3)-, and α-(1,6)-linkages (Stewart *et al.*, 1968), and is recognized primarily by Dectin-2 coupled with the Fc receptor gamma (Sato *et al.*, 2006; Saijo and Iwakura, 2011). A similar IgE-suppressive effect was exhibited by 2 yeast strains, zymosan, and mannan, suggesting that signal transduction via receptors for these components may be involved in IgE suppression.

S03 and P01 strains enhanced IFN-γ and IL-12 production in spleen cell cultures (Fig. 4A and 4B). At 100 μg/mL, zymosan showed stronger enhancement of IL-12 production than yeast strains did. Furthermore, the enhanced IL-12 p40 expression that was induced by S03 strain, P01 strain, and zymosan was shown in RAW264 cells, which were used as a model of macrophages, major producers of IL-12 (Fig. 5). Although mannan weakly induced IFN-γ production in spleen cells, it had little effect on IL-12 induction in both spleen cells and RAW264 cells. These results suggest that zymosan in S03 and P01 strains was involved in the inductive effect on IFN-γ and IL-12. IFN-γ and IL-12 are T helper (Th)1-type cytokines, which suppress Th2-type humoral immune responses and resultant IgE production (Ngan *et al.*, 1976; Kiniwa *et al.*, 1992). IL-12 is produced mainly by antigen-presenting cells, and is involved in the production of IFN-γ in T cells and NK cells (Chan *et al.*, 1991). The induction of IFN-γ and IL-12 expression suggests that the suppression of IgE production by S03 and P01 strains was mediated

by increased production of Th1 cytokines.

Fig. 3 shows that the effect of 100 μg/mL zymosan on IgE suppression was mitigated despite a strong inductive effect on IL-12 production in mouse spleen cells. These results suggest that other mechanisms also are involved in IgE suppression by wild yeast strains, in addition to the induction of Th1 cytokines. As shown in Fig. 5, the expression of IL-10 in RAW264 cells was induced by S03 strain, P01 strain, and zymosan. The magnitude of the induction by zymosan was apparently larger than that by S01 and P01 strains. IL-10 is known to be a suppressive cytokine that decreases IFN-γ production in Th1 cells (Fiorentino *et al.*, 1991). Dillons *et al.* (2006) reported that zymosan strongly induces IL-10 rather than IL-12 via TLR2- and dectin-1-mediated activation. Differences in total cytokine balance including IL-10, IL-12, and IFN-γ induced by the fungus body of S03 and P01 strains and zymosan may affect their IgE suppressive effect.

Commonly, type-I allergy is mediated by antigen-presenting cells presenting allergenic peptides, Th2 cells promoting Th2-type immune responses, and B-cells producing antigen-specific IgE. These IgE molecules bind high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils and cause type-I allergic symptoms by triggering several inflammatory immune reactions such as degranulation of effector cells, wherein FcεRI-bound IgE and specific multivalent antigen are clustered with specific antigens (Galli and Tsai, 2012). Therefore, the suppression of IgE production-mediating immune cells by interaction with probiotic microorganisms could be used as a strategy for the prevention and treatment of type-I allergies. From the study of Buts *et al.* (1990), the effect of probiotic *S. boulardii* on IgA production has been studied (Qamar *et al.*, 2001; Soylu *et al.*, 2008). Kourelis *et al.* (2010) recently reported that *S. cerevisiae* strains, other than *S. boulardii*, isolated from the human gastrointestinal tract enhanced IgA production in the mouse intestine. However, the effect of *Saccharomyces* strains, including *S. boulardii*, on IgE production remains unclear. Our study is the first, to our knowledge, to report the IgE-suppressive properties of wild *Saccharomyces* strains that were obtained from natural resources. Of course, the safety of these wild *Saccharomyces* strains *in vivo* will need to be individually and carefully evaluated in future studies to determine the safety of using *Saccharomyces* yeasts, including commercially available strains (de Llanos *et al.*, 2006; Klingberg *et al.*, 2008).

In conclusion, we demonstrated that wild strains of *S. cerevisiae* and *S. paradoxus* obtained from many natural resources can serve as useful candidates for application as IgE-suppressive food components. The mechanism of IgE-suppressive properties of isolated yeasts can be explained as an effect on increased Th1 cytokine production and improvement of the Th1/Th2 immune balance by inducing IL-12 production from macrophages, rather than as a direct effect on IgE production by plasma B cells. Further studies on the effects of wild yeasts are desired because of their potential beneficial effects in ameliorating type-I allergies.

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