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メタデータ	言語: eng 出版者: 公開日: 2019-12-20 キーワード (Ja): キーワード (En): baker's yeast, genetically modified microorganism, DGGE, microbial community 作成者: 安藤, 聡, 島, 純 メールアドレス: 所属:
URL	https://doi.org/10.24514/00002845

技術報告

Effect of a Genetically Modified Strain of Commercial Baker's Yeast on Microbial Communities in Simulated Natural Environments

Akira Ando and Jun Shima*

* National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki, 305-8642 Japan

Abstract

Disruption of the acid trehalase gene (*ATH1*) by genetic modification (GM) improves the freeze tolerance of baker's yeast, which is crucial for frozen-dough baking. We previously reported that *ATH1* disruption does not promote the survival of viable cells and DNA of baker's yeast in simulated natural environments. In this study, the effect of inoculation of a GM yeast strain on viable cell numbers and microbial communities of indigenous microorganisms in simulated natural environments was assessed by using the *ATH1* disruptant as a model GM yeast. The microbial community compositions were evaluated by using denaturing gradient gel electrophoresis (DGGE) of rDNAs. Changes in the number of viable cells and the DGGE band patterns of environmental samples inoculated with the GM strain were nearly the same as those inoculated with the wild-type (WT) strain, suggesting that the effect of the GM strain on microbial communities is not significantly different from that of the WT strain.

Key words: baker's yeast, genetically modified microorganism, DGGE, microbial community

Introduction

Genetic modification (GM) techniques for breeding baker's yeast *Saccharomyces cerevisiae* are well established. Characteristics such as fermentation ability and stress tolerance have been improved by using GM techniques[1-4]. Such improvements decrease the costs of baker's yeast production and of bakery processes. Therefore, GM techniques can be used in practical applications. However, the commercial use of GM strains is currently stalled due to a lack of scientific data on the survival of such strains in natural environments, as well as the effects of these organisms on the environment and on human health[5, 6]. A wide variety of yeast species have been detected in natural environments such as soil and water, and even strains of *S. cerevisiae* have been found in such environments[7, 8]. There is a need to assess the effects of GM yeasts on natural environments due to the potential for leakage of such yeasts into these environments. Such leakage might occur during the propagation process of yeast products in factories or during the leavening process in bakeries. It is important to provide the general public accurate information about the effects of GM yeast in order to promote its public acceptance in the commercial food industry.

We previously demonstrated that a GM yeast, which was an acid trehalase gene (*ATH1*) disruptant derived from commercial baker's yeast, exhibited a high accumulation of trehalose and improved freeze tolerance[1]. Based on that study, it is expected that commercial use of *ATH1*

*Corresponding author: Jun Shima (E-mail: shimaj@affrc.go.jp)

disruptants in frozen-dough baking would be effective[1]. We also previously assessed the survival of viable cells and DNA of *ATH1* disruptants constructed by GM and self-cloning techniques in simulated natural environments, and found that genetic modification of the *ATH1* locus apparently does not promote the survival of viable cells and DNA[9]. However, the effect of the *ATH1* disruptants on microbial communities was not studied at that time.

Despite the increased number of studies on GM plants and microorganisms[10-13], only a few studies on the behavior of GM yeasts in natural environments have been reported[9, 14, 15]. None of the studies have described the effect of GM yeasts on microbial communities in natural environments.

The aim of this study was to clarify the effects of GM yeasts on microbial communities in natural environments at the molecular level. For this purpose, the *ATH1* gene disruptant derived from commercial baker's yeast was used as a model of GM yeast, and was inoculated into two simulated natural environments (soil and water). To assess the effects of GM yeast on the microbial communities, changes in the number of indigenous microorganisms during 40 days were measured based on viable cell counts. The rDNA profiles of microbial communities in the inoculated environments were monitored by using DGGE (denaturing gradient gel electrophoresis) with PCR-amplified rDNAs as the samples. DGGE analysis is a cultivation-independent molecular analysis that has been used to profile complex microbial communities, including bacteria and fungi[16-18]. Here, we used these methods to conduct an environmental assessment of GM yeast at the microbiological level.

Materials and methods

Yeast strains

Prototroph diploid strains of *S. cerevisiae* T118CR-WT (*MAT***a**/α *ATH1/ATH1 cyh2/cyh2*) and T118CR-GM (*MAT***a**/α *ath1::kanMX4/ath1::kanMX4 cyh2/cyh2*) were used in this study. The strain T118CR-WT was a spontaneous cycloheximide-resistant mutant derived from commercial baker's yeast[9], and represented the wild-type (WT) strain in this study. The strain T118CR-GM was obtained from T118CR-WT by replacing the coding region of the *ATH1* gene with the kanamycin-resistant gene *kanMX4*[9], and represents the GM strain in this study.

Conditions of the simulated natural environments

The conditions of the two simulated natural environments (soil and water) were as follows. Horticultural non-sterile river sand (Matsuzaki™, Japan) was used as the model soil according to our previous study[9]. Sterile distilled water containing 10% (w/v) of the non-sterile sand was used as the model water. For the soil environment, each strain was inoculated into 70 g of soil in a 125-ml plastic bottle at a cell density of 10^6 cells/g of dry soil, and then was immediately mixed. For the water environment, each strain was inoculated into 500 ml of sand-containing water in a 1-liter flask at a cell density of 10^6 cells/ml of water, and then was immediately mixed. Both model environments were inoculated with either the WT or GM yeast cells grown at 30 ℃ for 48 h in YPD medium, and then was incubated at 25 ℃ for 40 d under dark conditions without shaking. During incubation, samples were taken every 5 d for viable cell counts and DNA extractions. At the time of sampling, the model environments were mixed to ensure homogenous samples.

Viable cell counts

The number of viable cells of inoculated yeast remaining in the soil and water environments was measured by using the plate count method as described previously[9]. In brief, the model environment samples were suspended in distilled water, and then plated onto CPS agar medium[9]. The CPS medium contained 5 μg/ml of cycloheximide and 0.75 mg/ml of sodium propionate to prevent the growth of indigenous fungi, including yeast. If the number of viable cells was expected to be less than 10^2 cells/g of soil or ml of water, the sample suspension was centrifuged to concentrate the viable cells. The number of colonies that appeared after incubation for 3 d at 30 ℃ on CPS medium was defined as the inoculated yeast viable cell number. To measure the number of indigenous bacteria, sample suspensions were plated onto tryptic soy agar (Difco Laboratory, USA) and then incubated at 37 ℃ for 3 d. The number of colonies that appeared on the agar was defined as the number of viable indigenous bacteria. To measure the number of indigenous fungi, the sample suspensions were plated onto rose bengal agar plates containing 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 5 g of pep-

tone (Difco), 10 g of glucose, 0.3 g of streptomycin, 33 mg of rose bengal, 20 g of agar (per liter), and then incubated at 25 ℃ for 5 d. The number of colonies that exhibited filamentous growth on the agar was defined as the number of indigenous fungi. The numbers of viable cells of bacteria, fungi, and yeast were expressed as the means of triplicate experiments.

DNA extraction

DNA extraction from the two simulated natural environments was conducted as follows. DNA contained in the soil environment samples (0.5 g) was extracted by using a FastDNA spin kit for soil (Q-Biogene, USA) as described by Takada-Hoshino and Matsumoto[19]. The extracted DNA was then dissolved in 50 μl of distilled water and used for PCR amplification as a template. DNA contained in the water environment samples (1 ml) was extracted as described by Davis et al.[20] with modifications. In brief, cells precipitated by centrifugation at 15,000 rpm for 3 min were incubated with 5 mg/ml of lysozyme (Siekagaku Kogyo, Japan) and 0.15 mg/ml of Zymolyase 100T (Seikagaku Kogyo), and then lysed with 0.4% SDS followed by the addition of 0.8 M potassium acetate. DNA in the supernatant of the lysate was ethanol-precipitated, dissolved in 50 μl of distilled water, and then was used for PCR amplification as a template.

DGGE analysis

DGGE analysis was performed according to Lopez et al.[21]. In brief, EX Taq-polymerase (Takara, Japan) and universal primers Ec 338 f-GC (5'-CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCCCCGCCCC ACTCCTACGG GAGGCAGCAG-3') and Ec518r (5'-ATTACCGCGG CTGCTGG-3') were used to amplify DNA fragments of bacterial 16S rDNA and fungal 18S rDNA by using PCR. The thermal cycling condition consisted of initial denaturing at 94 ℃ for 4 min, followed by 35 cycles of denaturing at 94 ℃ for 1 min, annealing at 54 ℃ for 1 min, and elongation at 72 ℃ for 1 min, and a final elongation at 72 ℃ for 5 min. DGGE was performed by using the DCode system (Bio-Rad, USA). The PCR product was loaded on 8% (w/v) polyacrylamide gels (1-mm thick) containing a linear gradient of 30 to 60% of denaturant, where 100% denaturant was 7 M urea and 40% (v/v) formamide. The gels were electrophoresed in 1 × TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA; pH 8.0) at 60 ℃ and 40 V for 15 h. The gels were stained by using ethidium bromide, washed twice with distilled water, and were examined by using UV transillumination.

Results and discussion

Changes in the number of viable cells in the soil environment

The changes in the number of viable inoculated yeast cells and indigenous microorganisms during 40 days in the soil environment were measured (Figs. 1A, B, and C). Figure 1A shows the viable cell count under control conditions, in which the model environment was not inoculated with yeast cells. Under the control condition, indigenous bacteria were detected at frequencies ranging from 1 × 10^7 to 5 × 10^6 CFU/g of dry soil throughout the entire 40-day incubation period. In contrast, indigenous fungi were detected at frequencies ranging from 8 × 10^2 to 1 × 10^2 CFU/g of dry soil throughout the entire 40-day incubation period. Neither the inoculation with the WT strain nor the GM strain significantly affected the numbers of bacteria or fungi (Figs. 1 B and C). These results strongly suggest that inoculation with WT or GM yeast strains did not affect the viability of indigenous microorganisms in the soil environment. Consistent with our previous observations[9], WT and GM yeast strains logarithmically decreased in a time-dependent manner.

Changes in the number of viable cells in the water environment

Similar to the results for the soil environment, the changes in numbers of viable indigenous bacteria and fungi in the simulated water environment were nearly identical for the WT, GM, and no-yeast control treatments (Figs. 1D, E, and F). These results suggest that inoculation with WT or GM yeast strains did not affect the viability of indigenous microorganisms in the water environment. In contrast to the comparable growth fitness of the WT and GM strains in the YPD medium, which were examined by using a growth competition assay as previously described[22] (data not shown), the number of viable cells of the GM strain decreased significantly more quickly than that of the WT strain in the water environment. This is consistent with our previous observations[9], which ex-

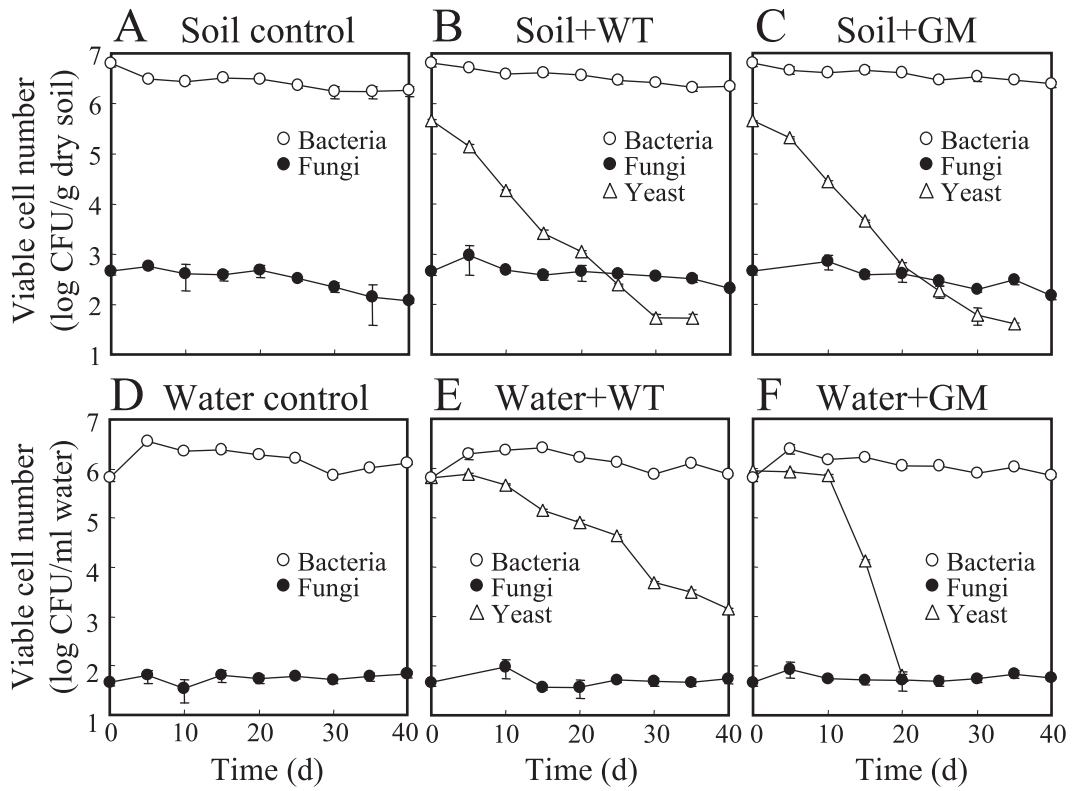

Figure 1.

Changes in the number of viable cells during 40-d cultivation in a simulated soil environment (A, B, and C) and water environment (D, E, and F) with and without inoculation of wild type (WT) and genetically modified (GM) yeast strains. Numbers of viable cells are expressed as means ± standard deviation from triplicate experiments.

amined the viability of co-inoculated WT and GM yeast strains in a water environment. The results suggest that the GM strain is less competitive than is the WT strain in a poor water environment.

DGGE analysis of the microbial communities in the soil environment

To gain further insight into the effects of GM yeast inoculation, we next examined the changes in rDNA profiles of the microbial communities in the soil and water environments by using DGGE analysis (Fig. 2). Figure 2A shows the changes in the DGGE pattern of PCR fragments amplified from DNA extracted from the soil environment. Almost all bands, such as bands A, B, and C, amplified from soil samples inoculated with either WT or GM strains, and from the no-yeast control, exhibited similar patterns throughout the entire 40-day incubation period. Although the intensity of band B increased transiently after 5 d of inoculation, the changes in patterns in GM strain-inoculated samples were similar to those in the WT strain-inoculated samples. Consistent with the viable cell count (Figs. 1B, C), bands representing the WT and GM yeast cells decreased in a similarly time-dependent manner (Fig. 2A). These DGGE data suggest that the effect of inoculation with the GM yeast strain was very similar to that observed with the WT strain, and that inoculation of neither strain affected the microbial community in the simulated soil environment.

DGGE analysis of the microbial communities in the water environment

Figure 2B shows the changes in DGGE patterns of PCR fragments amplified from DNA extracted from the water environment. In contrast to the case of the soil environment, the band pattern for no-yeast control samples was different from that for the yeast-inoculated samples. This result indicated that the microbial community in the simulated water environment was influenced by the inocu-

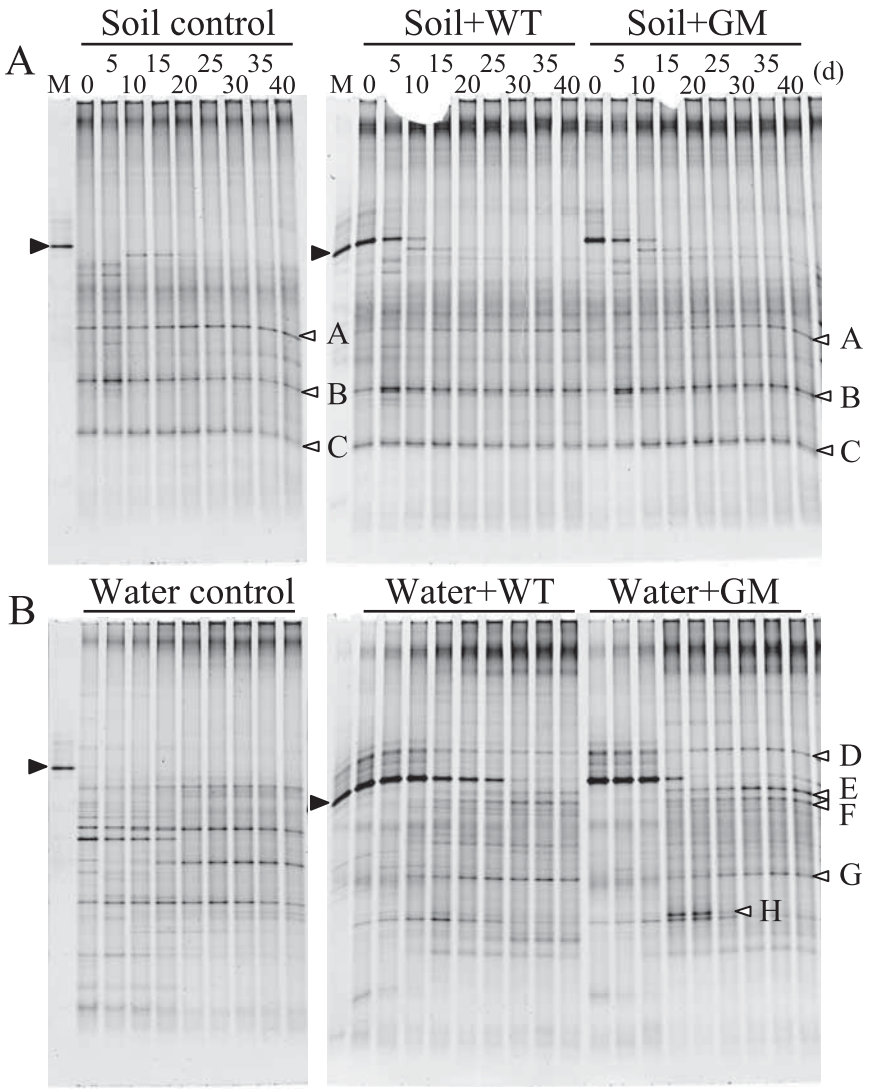

Figure 2.

Changes in microbial community compositions during 40-d cultivation in a soil environment (A) and water environment (B) analyzed by using rDNA-based DGGE profiling. The PCR product amplified by using genomic DNA from WT yeast cells was loaded on lane M as a control for rDNA fragment of baker's yeast (indicated by black arrowheads). White arrowheads indicate major bands.

lation with yeast. This could be simply explained by the assumption that the microbes in the simulated environment utilized nutrients derived from the dead cells of the inoculated yeast. As shown in Fig. 1, the simulated water environment exhibited a lower density of the viable bacteria and fungi compared with the simulated soil environment. Thus, the nutrient supply per microbial cell was relatively high in the simulated water environment, resulting in the changes in DGGE band patterns.

We next focused on the results from each yeast-inoculated sample. The intensities of bands D, E, F, and G were similar for the WT strain- and GM strain-inoculated samples throughout the entire 40-day incubation period. Although the intensity of band H for the GM strain-inoculated samples was higher than that for the WT strain -inoculated samples after about 20 d, this difference in intensity was transient. In fact, after 30 d, the H bands for both types of samples disappeared. These DGGE data suggest that the effect of inoculation with the GM yeast on the microbial community in the water environment was similar to that of the WT yeast. Consistent with our previous observations[9], the PCR fragments derived from the

GM yeast cells in the water environment decreased significantly more quickly than those from the WT yeast cells.

In the present study, the effects of a GM strain on both the total number of viable cells and on the composition of microbial communities in simulated soil and water environments were compared with those of the WT strain. Changes in the number of viable cells and in the microbial communities of GM strain-inoculated samples were nearly the same as those of WT strain-inoculated samples, suggesting that in these two simulated environments, there were no significant differences between the effects of inoculation with the WT and GM strains.

The present work suggests that inadvertent or intentional release of the GM yeast strain into natural environments will not affect the microbial communities. The survivability of the GM yeast strain was previously reported to be the same or lower than that of the WT strain[9]. Taken together, these results imply that the commercial utilization of GM yeast should have no negative effects on natural environments. However, the gene transfer from GM yeast to other organisms remains unknown. Further research is planned to study the horizontal gene transfer of genetically modified gene loci.

This is the first report of the effect of GM food microorganisms on natural environments. Other GM microorganisms used in the food industry, such as bacteria and fungi, will be developed in the near future. This study should be one of the advanced models for environmental risk assessment of GM food microorganisms.

Acknowledgments

This study was supported in part by a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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遺伝子組換え実用パン酵母が模擬的自然環境中の微生物集団に与える影響

安藤　聡，島　純

食品総合研究所

要　約

遺伝子組換えによる酸性トレハラーゼ遺伝子（*ATH1*）の破壊は，冷凍生地製パン法において重要であるパン酵母の冷凍耐性を向上させる．我々は以前に，*ATH1*破壊が模擬的自然環境中におけるパン酵母の生存や DNA 残存性を促進しないことを報告した．本研究では，遺伝子組換え酵母のモデルとして *ATH1* 破壊株を使用し，模擬的自然環境への遺伝子組換え酵母の接種が環境中の微生物集団に与える影響について検討した．微生物集団構成については，rDNA の変性剤濃度勾配ゲル電気泳動（DGGE）を用いて評価した．遺伝子組換え酵母を接種した模擬環境試料中の微生物生菌数および DGGE バンドパターンの推移は，野生型酵母を接種した試料中のそれと同等であったことから，各株が微生物集団に与える影響には顕著な差異はないものと示唆された．