土壌環境におけるAspergilus oryzae セルラーゼ高生産性組換え株及び宿主株の胞子生残性

食総研報（Rep. Nat'l. Food Res. Inst）No . 72, 53−58（2008）［報　文］

報　文

Survivability of conidia from a recombinant *Aspergillus oryzae* strain overproducing cellulase, and its parent strain in a soil environment

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Abstract

A recombinant *Aspergillus oryzae* strain TB1 produced by Kitamoto *et al*. (1999) overproduces cellulase 500−fold higher than its parental strain KBN616−39. Strain TB1 was reported to decrease soy sauce refuse, when it was used in production of soy sauce in a small scale experiment. To determine the survivability of the conidia of strain TB1 together with its parental strain KBN616−39 and a wild type strain PTR−1, they were inoculated in black soil packed in plastic tubes as a soil environment and incubated at 25℃. Colony formation was determined using the dilution plating method. Repetitive survivability experiments showed that the average survivability−decreasing rates and dispersion of strain TB1 were the same as strains KBN616−39 and PTR−1, where they survived over seven weeks at 25℃ in the soil environment. None of the strains showed an increase in colony formation. Thus, we suggest that their conidia neither germinate nor grow in soil.

Introduction

Aspergillus oryzae is a filamentous fungi used in East Asian countries as *koji* molds in the manufacture of various fermented foods such as soy bean paste, soy sauce, rice wine and also in the production of food−grade and pharmaceutical enzymes worldwide. Recent progress in the recombinant DNA technique for *A. oryzae* has led to the development of recombinant strains for practical use. For instance, a strain carrying the gene for glucoamylase under the control of a strong promoter (*melO*, a gene for tyrosinase) was developed, and it produces large amounts of the enzyme, e.g., at the level of 0.8 to 3.3 g per liter of broth[1)]. Another example is *A. oryzae* strain TB1, which was derived from a strain used in soy sauce production. This strain carries multi−copies of the cellulase (endo−1,4−beta−glucanase) gene, and produces this enzyme up to about 500−folds, compared to its host strain in the appropriate media[1)]. Such strains could be beneficial if used in industry. Kitamoto *et al*. (1999) described that the use of strain TB1 in a small−scale fermentation experiment of soy sauce, decreased soy sauce refuse, a kind of food −derived waste, and that it increased filtration efficiency of soy sauce mash[3)]. The development of strain with such a high degree of enzyme production may only be established by the recombinant DNA technique, and not by the general mutagenesis technique. Before such useful recombinant stra s these recombinant strial use, however, we first need to describe the environmental effect these recombinant strains may habe if acciden−

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tally released into the environment. *A. oryzae* strains used for soy sauce produce a large amount of conidia. Therefore we should examine and compare the survivability of conidia from strain TB1 and its parental strain KBN616-39. In general, survival and persistence study methods for recombinant microorganisms may include one of the following ecosystems: activated sludge, mammalian gut, soil, or river water[4]. For instance, data has shown a rapid decrease in the viability of recombinant *Escherichia coli* in river water[5]. Other data described low viability of recombinant *Saccharomyces cerevisiae* in a soil environment[6]. We know little about the survivability in the natural environment of even non-recombinant *A. oryzae* strains that are used in manufacturing. Moreover, a method to assess the survivability of *A. oryzae* has not yet been established.

A. oryzae is thought to have been isolated from nature in ancient times, and was domesticated by manufacturers[7]. The fungi's natural environment and its tolerance to it are of great scientific interest. Suzuki *et al*.[8] reported that fungus belonging to *A. oryzae* was detected from the fungal community (fungal ball) — sometimes found on the ears of rice cultivated in fields — so-called *ina-koji* ('ina' means rice plant). According to their paper, the main species in this community was *Ustilaginoidea virens*, and *A. oryzae* was only a minor species. Moreover, they found that *A. oryzae* was detected from ears, leaves, and the straw of rice plants, and in the soil of rice fields[9]. These findings suggest the strong survivability of *A. oryzae* and their growth in the environment. Besides these wild type strains, those used in industry are thought of as house-keeping strains. Therefore, their effect on the environment, and the effects of their derived recombinant strains, could be different from those of wild type strains. Assessment of the effect of the recombinant organisms on the environment is generally performed by comparing the survivability of the recombinant one with the non-recombinant host. In this study, we therefore focused on the survivability of conidia from strain TB1 in a soil environment, together with two non -recombinant *A. oryzae* strains, one of which is commonly used in soy sauce production (KBN616-39) and one derived from a wild type strain (PTR-1).

Materials and Methods

Fungal strain and growth conditions

A. oryzae strains TB1, KBN616-39, and PTR-1 were used in this study. KBN616-39 was a *niaD*$^-$ mutant strain derived from KBN616, which is used in the production of soy sauce. TB1 was a strain obtained by cotransformation of KBN616-39 with the plasmid pTACB100 carrying the *celB* gene (encoding endo-1,4 -β-glucanase) under the control of the Taka-amylase gene (*AmyB*) promoter and the plasmid pND300 carrying the *niaD* gene encoding nitrate reductase[2]. PTR-1 was derived as a pyrithiamine-resistant strain from NFRI1599 (RIB40), which was first isolated from cereal. These strains were grown on malt extract agar plate media for 14 days at 25℃. Each conidia suspension was prepared as follows. Conidia were scraped from the agar surface by adding 20 ml of sterile water to the plate ; then they were filtered through Miracloth (Calbiochem, CA, USA). The conidia suspension was put into a 50-ml centrifuge tube, centrifuged for 20 min at 0.86 x g (Hitachi Koki, himac CR22E, Hitachi, Japan), and was rinsed once with 20 ml of sterile water. The suspension was again centrifuged as above and finally 10 ml of sterile water was added to prepare the conidia suspension.

Survivability test in the soil

Black soil for gardening was obtained commercially (Green Tamadaya, Kanuma, Japan). The water content of the soil was 44 % (w/w). The soil was kept at room temperature until use. The soil was divided into 2 g portions that were put in disposable 50 ml plastic tubes. Conidia suspensions containing 2 x 10^5 of conidia were inoculated into the soil in each of the plastic tubes. They were then covered with lids and incubated at 25℃. All the samples were weighed at the beginning of the experiment, and subsequently weighed every week to check the water content. If the sample weight decreased by more than 0.3 g, the weight loss was compensated for by adding sterile water. Sampling of the soil was done periodically from each of the three

tubes by adding 10 ml of distilled water into each tube. The tube was then sonicated (Branson, Model 1210, degassing mode) for 10 min and vortexed to extract the microorganisms. The appropriate volume of the diluted sample was spread on a modified Czapek-Dox agar plate medium containing streptomycin with ammonium chloride as a nitrogen source (10 g l^{-1} glucose, 6 g l^{-1} NH_4Cl, 0.52 g l^{-1} KCl, 1.52 g l^{-1} KH_2PO_4, 2 mmol l^{-1} $MgSO_4$, 1 mg l^{-1} $FeSO_4$, 8.8 mg l^{-1} $ZnSO_4$, 0.4 mg l^{-1} $CuSO_4$, 0.1 mg l^{-1} $Na_2B_4O_7$, 0.05 mg l^{-1} $(NH_4)_6Mo_7O_{24}$, 40 mg l^{-1} streptomycin, 20 g l^{-1} agar, pH not adjusted). For identification of strain PTR-1, 0.1 ng ml^{-1} pyrithiamine was added to the media. The number of visible colonies was counted after incubation for two days at 25℃, and the colonies whose morphological character was the same as the inoculated *A. oryzae* strain were regarded as surviving *A. oryzae*. The number of viable *A. oryzae* was estimated by the dilution plating method and was calculated per gram of soil, using at least three incubated plates for each extracted sample. The survivability of each *A. oryzae* strain was shown as the ratio of the number of detected *A. oryzae* colonies at the time of sampling against the number at inoculation.

Results and Discussion

We examined the survivability of one recombinant and two non-recombinant strains of *A. oryzae* in the black soil. Each survivability experiment was done twice or three times. Incubation of the conidia from the three strains of *A. oryzae* was continued for 42 days, except one experiment for strain TB1 which continued for 41 days, and one for strain PTR-1 which continued for 43 days. Initial *A. oryzae* colonies detected on the agar plate media were from about 3000 up to 5000 cfu/g soil. Identification of the inoculated *A. oryzae* colonies was easy, as there was no fungal colony resembling *A. oryzae* derived from the soil when *A. oryzae* was not inoculated into the soil (data not shown). Survivability for all experiments was calculated using the mean of the three soil samples with the standard deviation at each sampling point. The survivability versus incubation time was plotted for each test. The results are shown in Fig. 1. During these periods, survivability of all the tested *A. oryzae* strains in the soil decreased with fluctuation. Some of the data points included relatively a large deviation (up to 34 % of the average number). To compare the survivability-decreasing rate, we hypothesized that the decreasing pattern tended to a simple linear manner. Then we performed linear regression analysis against each series of the data. The linear regression coefficient is shown in Table 1. The linear regression coefficient varied between −0.65 and −0.97. These numbers showed a relatively strong inverse correlation between the survivability and the incubation time. Therefore the survivability of the conidia for the three strains in the black soil, during the tested period, may approximately follow the linear regression line whose formula is shown in each graph in Fig. 1. The slope of the regression line, i.e. the survivability-decreasing rate, is compared in Table 1. Those for strain TB1 (three tests) were −0.41 through −0.82. Those for strain KBN616-39 (three tests) were −0.55 through −0.79. Those for strain PTR-1 (two tests) were −0.57 and −0.64. Due to the large dispersion among each data set within each survivability test, especially with the strains TB1 and KBN616-39, we could not perform the statistic analysis if the survivability-decreasing rates of the three strains were intrinsically the same or not. Hypothesizing that those data included only experimental error and had no effect from other factors such as microorganisms living in the soil, the average of the survivability-decreasing rate could be calculated as −0.64 for strain TB1, and −0.66 for strain KBN616-39. These average numbers were close to each other and to the rate for strain PTR-1. Since PTR-1 was a derivative of the wild type strain of *A. oryzae*, it was possible that the house-keeping strain KBN616-39 had the same extent of survivability in the tested soil as the wild type strain. It was also suggested that both had the same extent of survivability and dispersion for the recombinant strain TB1 and the non-recombinant strain KBN616-39. If the survivability-decreasing rate of the tested strains was constant over the period we examined, the tested *A. oryzae* strains may survive for four to eight months in the tested soil environment. None of the tested strains showed an increase in colony formation. Thus, we suggest that

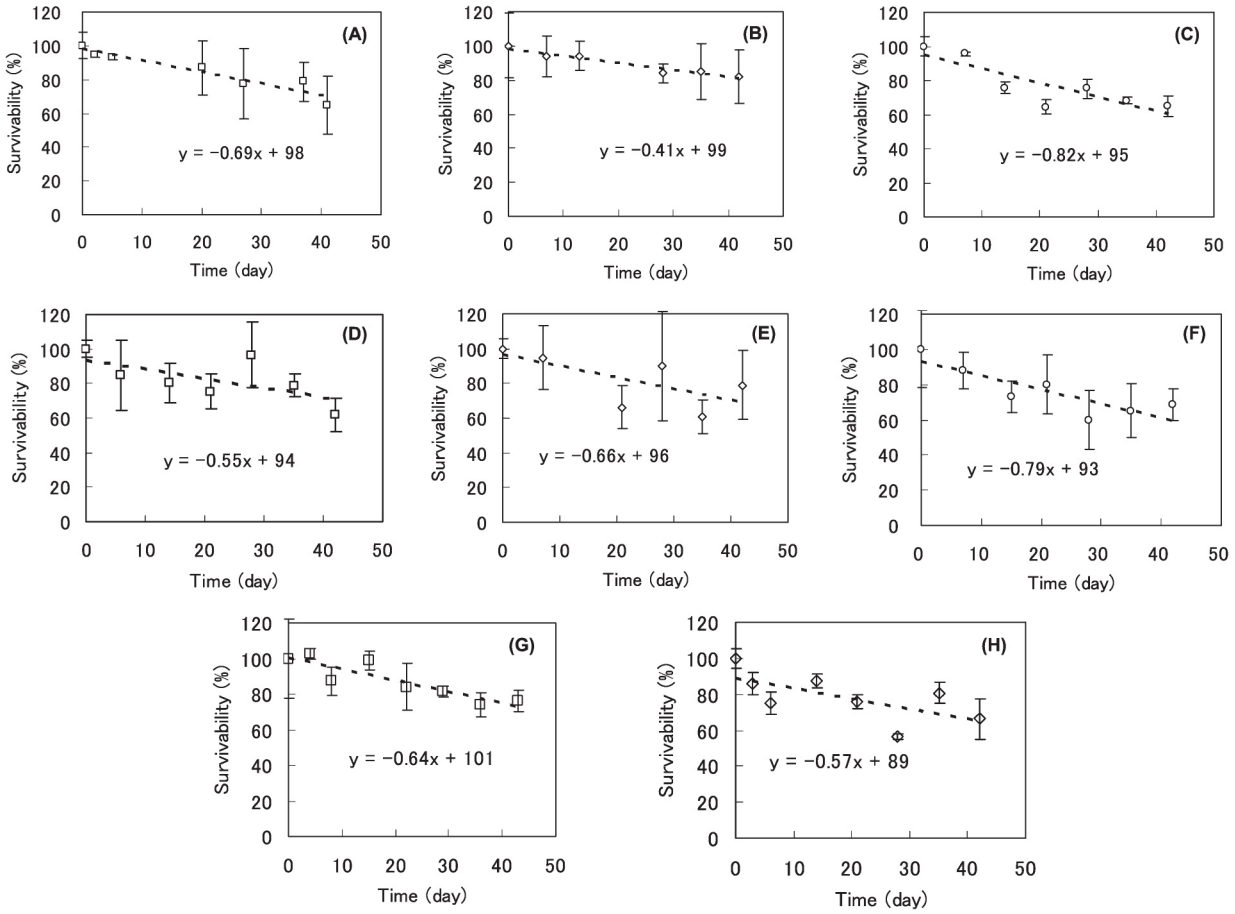

Fig 1. Change in the survivability of strains TB1, KBN616-39, and PTR-1

Three independent tests were performed for each strain. Graphs A-C, TB1 ; D-F, KBN616-39; G and H, PTR -1. The experiment with A was performed from December 17, 2003 through January 27, 2004. For B it was performed from April 13, 2004 through May 25, 2004. For C it was performed from June 8, 2004 through July 20, 2004. For D it was performed from February 10, 2004 through March 23, 2004. For E it was performed from April 19, 2004 through May 31, 2004. For F it was performed from July 5, 2005 through August 16, 2005. For G it was performed from June 16, 2003 through July 28, 2003. For H it was performed from September 16, 2003 through October 28, 2003. Each datum point is the mean of three independent samples. Bars represent the mean ± SD of the three measurements. The dotted line presents the approximate linear line according to the results of the linear regression analysis. The formula for linear regression is shown in each graph.

their conidia do not germinate or grow in black soil.

The number of the colony-forming fungi other than the inoculated *A. oryzae* strain in the soil was initially 500 to 800 cfu/g soil (data not shown). Their change seemed independent of the inoculated strains, KBN616 - 39 or TB1 (data not shown). Bacterial and yeast colonies were not detected on the agar media used in this study. Whether the inoculated *A. oryzae* had any effect on the native fungi in the soil sample needs further study. Note that the data presented here regard-ing the survivability of the recombinant *A. oryzae* strain TB1 was the first report in this area of research.

In view of the applicability of genetically modified *A. oryzae* in the future, its accidental release into the environment should be considered. This study was performed as a first step in determining the effect of released recombinant *A. oryzae* (mainly conidia) on the soil environment, including its impact on native microorganisms in the soil surrounding manufacturing plants. Suzuki *et al*[8]. demonstrated that *A. oryzae* were present in rice fields. Their result suggested a high level of survivability of wild type *A. oryzae* in the outside soil. *Aspergillus flavus or Aspergillus par-asiticus*, related species of *A. oryzae*, are ordinarily

Table 1. The comparison of the linear regression coefficient and the slope of the regression line for the three tested strains

Strain	Linear regression coefficient	Slope (% survivability decreasing per day)
TB1		
Test 1	−0.95	−0.69
Test 2	−0.97	−0.41
Test 3	−0.86	−0.82
KBN616-39		
Test 1	−0.65	−0.55
Test 2	−0.67	−0.66
Test 3	−0.85	−0.79
PTR-1		
Test 1	−0.89	−0.64
Test 2	−0.67	−0.57

found in corn, peanut, and cotton fields[10) 11)]. Likewise, they should also retain a high level of survivability in the field. On the contrary, the black soil used in this study most likely contained a different microorganism community. However, our results also showed high survivability of our tested *A. oryzae* strains. The strain *A. oryzae* PTR-1 was derived from NFRI1599, which was originally isolated from cereal in 1950. The strain PTR-1 is therefore considered a wild type strain. In contrast, strain KBN616-39 and its recombinant strain TB1 were derived from strain KBN616, which is used specifically in producing soy sauce. It is not clear to what extent the genetic background of these two strains (NFRI1599 and KBN616) resembles that of the wild type *A. oryzae* strain detected in rice fields by Suzuki *et al.*[7)]. Strains PTR-1 and KBN616-39 have different morphological characteristics and different derived sources. Nevertheless, they showed the same extent of survivability in our study.

In this study, we proved that conidia of the three strains of *A. oryzae* retained their survivability for several weeks in the tested black soil environment. We also showed that the survivability-decreasing rate of a recombinant strain TB1 and its parent strain KBN616-39 were similar.

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土壌環境における *Aspergilus oryzae* セルラーゼ高生産性組換え株及び宿主株の胞子生残性

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要　旨

麹菌として用いられる *Aspergillus oryzae* は酵素生性が高く，酵素工業的に応用されている．近年組換え DNA 技術により，各種高生産株が作出されている．*A. oryzae* TB1 株は，醤油麹菌宿主株 KBN616-39 株から作出され，宿主の 500 倍のセルラーゼを高生産する菌株である．TB1 株，KBN616-39 株，及び野生株 PTR-1 株の胞子の土壌中の生残性を比較するため，プラスチックチューブを用いて，黒土にこれらの胞子を接種し，25℃で保温した．経時的に，希釈平板法により形成コロニー数を計測し，*A. oryzae* 生菌数を決定した．生残試験を反復した結果，TB1 株の生残率減少速度の平均及び分散は KBN616-39 株及び PTR-1 株と同程度であった．これらの菌株は，供試土壌環境中で 25℃において 7 週間以上生残した．生菌数が増加する菌株は見られなかったことから，供試した土壌中では，供試菌株の胞子は発芽生育しないと考えられた。