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Original Article

The Nucleotide Sequence Determination of Catalases of Three Medically Important Yeasts Using Newly Designed Degenerated Primers

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Abstract

We have developed degenerated primers for the isolation of several fungal species catalases, based on the known catalase genes of several yeast species. Using a combination of degenerated primers and the nested polymerase chain reaction (PCR) method, we were able to obtain PCR products from *Candida dubliniensis*, *C. tropicalis*, and *C. glabrata*. The nucleotide sequence of the PCR products amplified showed that those fragments contained sequences homologous with the known *Candida* catalases, indicating the usefulness of the designed primers. We determined the nucleotide sequences of the open reading frames and respective 5' untranscribed regions of these yeasts and compared each sequence with that of the respective related species. The difference between the deduced amino acid sequence of catalase of *C. dubliniensis* and *C. albicans* was 5 in 485 amino acids. The nucleotide sequence of *C. glabrata* catalase was identical to the sequence results from the genome sequence project which was recently released, whereas that of the catalase of the *C. tropicalis* clinical isolate was not the same as the strain Pk233, n-alkane-utilizing *C. tropicalis*.

The catalase activities of all the strains tested so far were activated by short-term hydrogen peroxide treatment, suggesting that common mechanisms were involved in the induction of catalase activity, although the nucleotide sequences of the 5' untranscribed region of these yeasts were diversified.

Key words: *Candida dubliniensis*, *Candida tropicalis*, *Candida glabrata*, catalase, reactive oxygen species

Introduction

The reactive oxygen species (ROS), which are highly toxic agent for cellular components such as proteins, lipids, or nucleic acids, are inevitably produced as a by-product during the process of oxygen respiration[1]. To prevent injury of the components by ROS, the oxygen-utilizing organisms are equipped with several types of ROS-detoxyfying enzymes. In addition, in the human immune system, phagocytes release the ROS to kill pathogenic microorganisms that have invaded the host body[2]. Escape from such an attack is needed for a microorganism to establish infection. Therefore, for pathogenic microorganisms, the ROS-detoxifying enzyme is very important to protect them not only from internal ROS but also from external ROS.

Catalases are enzymes that decompose hydrogen peroxide to molecular oxygen and water. It is known that catalases of some human infectious bacteria play a significant role in the establishment of infection[3,4]. In eukaryotic infectious microorganisms, the catalase has been proven to contribute to pathogenicity[5,6]. However, little is known about the characteristics of catalases of pathogenic fungi except for those of *Candida albicans*[6,7] and *Aspergillus* spp.[8]. To clarify the significance of catalase action during pathogenicity against a host, we must gain a comprehensive understanding of genetic regulation of the catalase activity in pathogenic fungi. We recently designed degenerated primers to obtain the nucleotide sequence of the fungal catalases, and developed procedures for isolating the catalase

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genes from several pathogenic yeasts using these primers.

Here, we report the complete nucleotide sequences of three yeast catalases, including the 5' untranscribed region. We also show that the messenger RNA of the catalase of these yeasts is induced by short-term exposure to hydrogen peroxide, which is same as occurs in known pathogenic yeasts.

Materials and Methods

Strains, media, and growth conditions

Candida dubliniensis IFM48313, *C. glabrata* TIMM1064, and *C. tropicalis* NUM5076 were used. *C. albicans* CBS9120 was used as a reference strain. YPD medium (2% glucose, 2% bacto-peptone (Difco, the U.S.A.), 1% bacto-yeast extract (Difco)) was used as a routine culture medium, and cells to be subjected were grown at 30°C with gentle shaking.

Polymerase chain reaction (PCR)

The KOD dash DNA polymerase (Toyobo, Japan) and its accompanying buffer were used throughout the experiment. We followed the manufacturer's recommendation regarding the general PCR conditions. When the degenerated primers were used, 0.1 μmole of forward and reverse primers were added to the 25 μl reaction mixture, and the PCR annealing condition was 44 or 45°C. In the 2nd (nested) PCR, a reaction mixture of the 1st PCR diluted a hundred-fold with DW was used as a template. The amplified DNA was purified from products of the 2nd PCR (100-200 μl) using Ultra Clean 15 (Mo Bio Laboratories Inc., the U.S.A.), and its nucleotide sequence was determined by ABI prism 310 (Applied Biosystems, the U.S.A.).

Construction of the genomic cassette library and PCR amplification of the unknown region next to the known sequence

Genomic DNA was prepared from cells grown in 2 ml YPD by the method of Holm *et al*[9]. Five micrograms of the DNA was digested with a restriction enzyme (for example, *Eco* RI) and then ligated with the cassette (LA-PCR *in vitro* cloning kit, Takara shuzo, Japan) having the same restriction site at one end (for example, *Eco* RI). Procedures for amplification of the unknown region next to the known sequence were described previously[7]. When the direct sequence analysis was not successful, probably due to partial heterogeneity of the PCR products, we cloned the products into DH5α bacterial strain using the TOPO TA cloning kit (Invitrogen, the U.S.A.) and followed by sequencing procedure.

Preparation of total RNA and Northern blot analysis

Total RNA was isolated by the method of Köhrer and Domdey[10]. About 20 μg of total RNA of each strain was run on denatured agarose gel, followed by blotting to positively charged nylon membrane Gene Screen Plus (Perkin Elmer Life Science, Inc., the U.S.A.). A species-specific probe of catalase was prepared by PCR amplification with 12.5 nmole of digoxigenin (DIG)-conjugated dUTP (Roche Diagnostics, Switzerland). Procedures for hybridization and detection of the DIG-labeled catalase followed the manufacturer's instructions.

Measurement of the catalase activity

Procedures for the extraction and measurement of catalase activity were described previously[7].

Results

Design of degenerated primers for the fungal catalase gene

The yeast catalases including *C. albicans* are composed of a small subunit of a single polypeptide (50-60 kDa, ref. 11) and contain no introns in the genomic sequence. Among the known sequences of yeast catalases, there are two conserved regions in the amino acid sequences, a heme binding region and an enzymatic active site region. Klotz *et al.* called this portion between two regions the core region and they performed phylogenetic analysis using this region[11]. We therefore assigned the primers to heme-binding and active site regions (Fig. 1). By comparing nucleotide sequences of 8 catalase genes from 7 yeast species, we decided the primer position and designed the degenerated primers as shown in Fig. 1 and Table 1. The degree of degeneracy calculated from the number of mixed nucleotides ranged from 256 to 1728. Although a distinct band was not observed in the 1st PCR, we could detect one or two bands in most of 2nd PCR amplifications. The representative amplification pattern in the 2nd PCR is shown in Fig. 2.

Comparison of the *Candida dubliniensis* catalase gene with the *C. albicans* catalase gene

C. dubliniensis is frequently isolated from AIDS patients and was described as a new species in 1995[12]. The physiological and genetic characteristics of *C. dubliniensis* are closely related to those of *C. albicans*: for example, both species form a germ tube in a serum-containing medium.

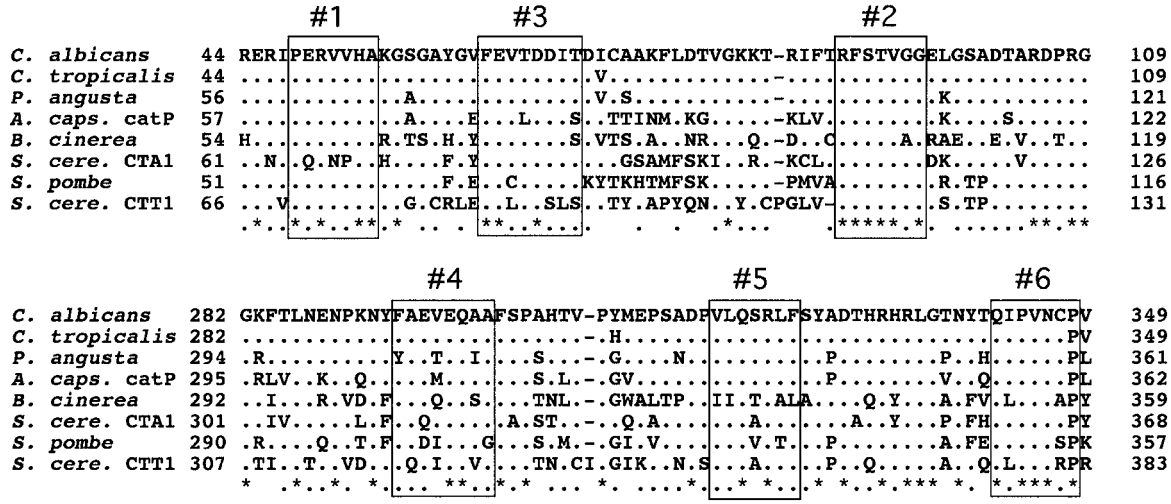

Fig. 1. Alignment of amino acids from 8 catalases of 7 yeast species.
Alignment among the sequences was taken using the software GENETYX-MAC (Software Development, Japan). The degenerated forward (#1, #2, and #3) primers and reverse (#4, #5, and #6) primers were designed based on the nucleotide sequences of respective regions. *P. angusta*; *Pichia angusta*, *B. cinerea*; *Botrytis cinerea*, *S. cere.*; *Saccharomyces cerevisiae*, *S. pombe*; *Schizosaccharomyces pombe*.

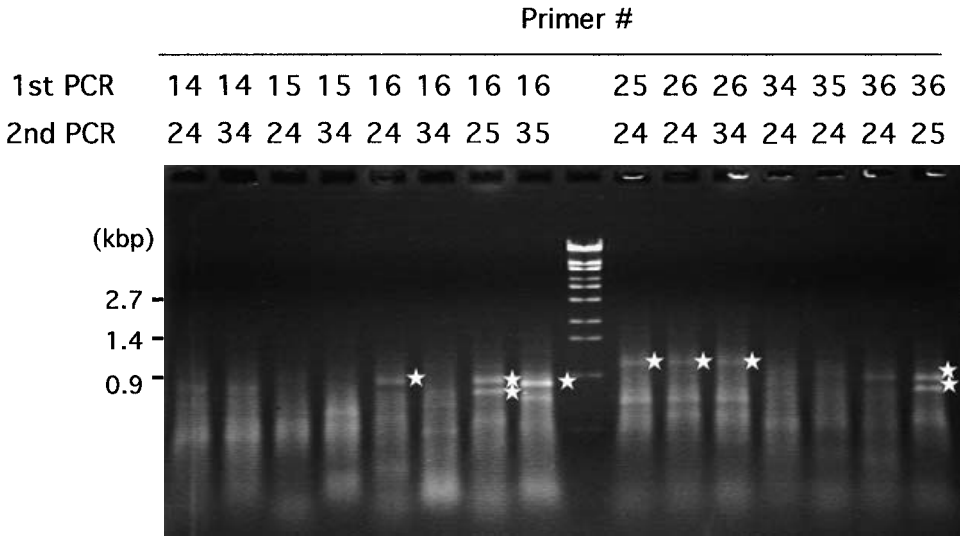

Fig. 2. Amplification patterns of the 2nd (nested) PCR using *C. tropicalis* as a template.
Each primer number indicates the combination of the forward primer and the reverse primer. For example, on the left-most column the #1 and #4 primers were used in the 1st PCR and the #2 and #4 primers were used in the 2nd PCR. Detailed procedures are described in Materials and Methods. The distinctive bands indicated with the asterisks were excised from the gel and their nucleotide sequences were determined.

Table 1. List of degenerated primers used in this study

	Primer #[a]	Sequences[b]	Degeneracy
Forward primers	#1	CCNGARMGNGYBGTYCAYGC	1536
	#2	CGNTTYTCBACBGTBGSNGG	1728
	#3	GARGTNACRGAYGAYATNAC	256
Reverse primers	#4	YTGYTCVRTYTCBGCRAARWA	1152
	#5	TGYTCVRTYTCBGCRAARSA	576
	#6	GGRCARTTNACNGGNATYTG	512

[a]: The primers #3 and #6 were the generous gift of Dr. N. Mutoh, Aichi Human Service Center, Kasugai, Aichi, Japan.
[b]: Characters other than A, T, C, and G indicate mixed nucleotides; B, T+C+G; M, A+C; N, A+C+G+T; R, A+G; S, C+G; V, A+C+G; W, A+T; Y, C+T

To compare the open reading frame (ORF) region and the 5' untranscribed region of both species, we amplified the core region of *C. dubliniensis* catalase using the nested PCR method, and then the neighboring regions were amplified using the cassette library, as described in Materials and Methods.

The homology score between *C. dubliniensis* catalase (GenBank/EMBL/DDBJ accession no.: AB181389) and *C. albicans* catalase was 96.0% (1399/1458) in nucleotides and 99.0% (480/485) in amino acids. Of 59 nucleotides substituted, 53 involved changes in the third letter of the

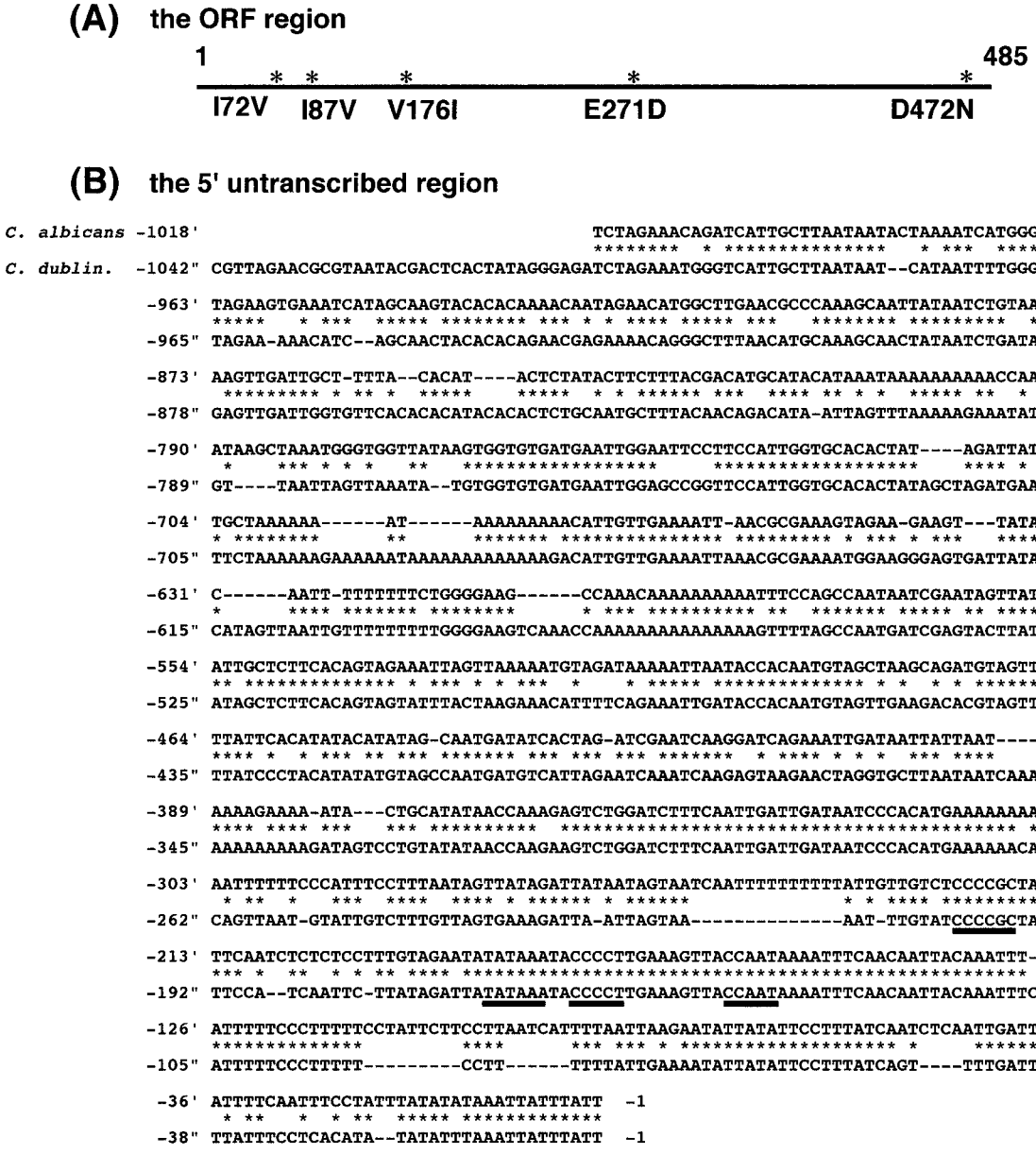

Fig. 3. Comparison of sequences of catalases of *C. dubliniensis* with that of *C. albicans*.

(A) Comparison of amino acids between two catalases. Asterisks indicate tha positions of amino acid substitutions. The I72V indicates that isoleucine at position 72 in *C. albicans* is substituted with valine in *C. dubliniensis*.

(B) Comparison of the 5' untranscribed region between two catalase genes. Alignment between two sequences was taken using the software GENETYX-MAC (Software Development, Japan). The nucleotide position was numbered as an ATG (initiation of the open reading frame) relative to 1. Hypothetical transcriptional regulatory motifs were indicated by underlines (7). *C. dublin.*; *Candida dubliniensis*.

genetic codon and did not change the amino acid. All the changes in amino acids were substitutions to related amino acids: 3 of 5 substitutions were valine to leucine or *vice versa*, one was aspartic acid to glutamic acid, one was aspartic acid to asparagine (fig. 3(a)). On the other hand, the homology score of the untranscribed region (about 1 kbps) of the two yeasts' catalases was 71.1%. In spite of the relatively low homology of the entire untranscribed region, sequences that had been considered hypothetical transcriptional regulatory motifs such as the stress responsive element or the hypothetical MIG1 binding motif in *C. albicans* did exist in *C. dubliniensis* (Fig. 3(b)).

Comparison of *Candida tropicalis* clinical strain from *C. tropicalis* Pk233 strains

The nucleotide sequence of the catalase of *Candida tropicalis* was released in 1989, which was the earliest description of a *Candida* catalase gene[13,14]. The strain Pk233 is characterized by *n*-alkane utilizing yeast, and catalase activity was induced under some culture conditions[15]. Recently, Kato *et al.* reported phylogenetic relationships

among the DNA topoisomerase II genes in *Candida* species[16]. They described that the nucleotide sequences between *C. tropicalis* Pk233 and clinical strains were too divergent to be in a single species. To determine whether this is also the case in the catalase gene, we amplified the core sequence of the catalase gene of *C. tropicalis* clinical strain NUM 5076[16] and determined the whole nucleotide sequence (GenBank/EMBL/DDBJ accession no.: AB181391). Homology between the ORF regions of the two catalases was 84.5% in nucleotides and 94.6% in amino acids. These are low scores as compared with the value between catalase sequences of *C. albicans* and *C. dubliniensis*, which are species discrete from each other. Moreover, no significant homology was observed in the 5' untranscribed regions of the two strains (data not shown).

Comparison of the *C. glabrata* catalase gene with the *S. cerevisiae* catalase genes

It has been shown that *C. glabrata* is a more closely related species to *S. cerevisiae* than any other pathogenic *Candida* species[16]. *S. cerevisiae* has two types of catalase, *CTA1* and *CTT1*[17, 18]. The two catalases differ in sequence, localization, and transcriptional regulation[17–21]. To know which type of catalase *C. glabrata* harbors, we amplified the core region of the catalase of *C. glabrata* using a set of degenerated primers. After determination of the whole sequence of the catalase gene, including the 5' untranscribed region, we compared the deduced amino acid sequence with that of other known fungal catalases. We found that the *C. glabrata* catalase (GenBank/EMBL/DDBJ accession no.: AB181390) was more closely related to the *S. cerevisiae CTA1* sequence, than to the *CTT1* sequence, as shown in Table 2. However, *C. glabrata* catalase is different from *S. cerevisiae CTA1* catalase in several of its amino acid and nucleotide sequences features.

Table 2. Homology of the catalase sequences determined in this study with the known sequences of yeast catalases

Species (accession nos.)	a.a.	homology (%)		
		C. tropicalis (485 a.a.)	*C. dubliniensis* (485 a.a.)	*C. glabrata* (507 a.a.)
C. albicans FC18 (AB006327)	485	**93**	**99**	70
C. tropicalis Pk233 (X06660)	485	**95**	**94**	70
S. cerevisiae CTA1 (X13028)	515	67	68	**81**
CTT1 (X04625)	573	56	57	**51**

Homology scores compared in this paper are emphasized by large font.

One difference is that the PTS (peroxisome targeting signal) motif (typically S-K-L in amino acid sequence), which is shown in the C terminus of the *CTA1* sequence and is involved in localization of this enzyme in peroxisome[21], is not observed in the *C. glabrata* sequence. The ORE (oleate-response element) and the ADR (alcohol dehydrogenase regulator) motifs are located in the 5' untranscribed region of the *CTA1* gene [21, 22]. We could not find either motif in the 5' untranscribed region of the *C. glabrata* catalase gene. Instead, several STRE (stress responsive element, CCCCT or AGGGG) sequences, which are located in the 5' untranscribed region of the *CTT1* gene in *S. cerevisiae*[23], were detected in the 5' untranscribed region of the *C. glabrata* catalase gene. These results suggest that the hypothetical regulatory component(s) of the *C. glabrata* catalase is similar to that of *CTT1*, although the primary sequence of its ORF is similar to that of *CTA1* at least in the homology score. Recently, the entire genomic sequence of *C. glabrata* was released on the website (URL: http://cbi.labri.fr/Genolevures/elt/CAGL). According to this information, nucleotide sequence of the catalase is identical of our results including the 5' untranscribed region (data not shown).

Response of the catalase genes against hydrogen peroxide

All the catalases studied in this paper are derived from human pathogenic yeast strains. When these yeasts invade the human body, they are challenged by phagocytotic cells such as neutrophils or macrophages by releasing ROS to kill pathogens. To survive such an environment, the yeasts must quickly synthesize catalase to detoxify ROS. To determine whether catalases are induced immediately after treatment of hydrogen peroxide, we performed Northern blot analysis and measured the catalase activity. As shown in Fig. 4, all the strains used here responded to the treatment of a low concentration of hydrogen peroxide.

Discussion

We have designed a set of degenerated primers that can amplify a core region of the fungal catalase genes. The primers were designed based on the knowledge that the heme binding site and the active site for enzyme reaction are well conserved among the catalase genes. Using these primer sets, we could amplify core regions of the three *Candida* species and determine the entire nucleotide sequences of catalase genes of

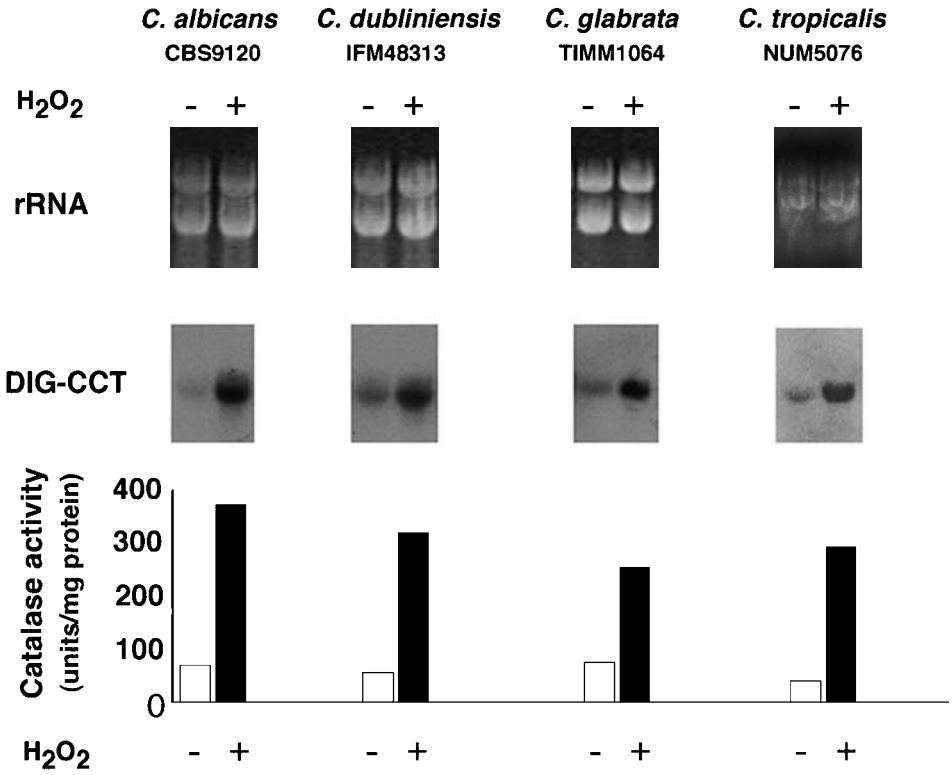

Fig. 4. Response of the catalase gene and activity upon the treatment with a hydrogen peroxide.
Each early logarithmic culture was treated or untreated with 0.5mM (final concentration) of hydrogen peroxide for 30 min. Before collecting the cells, each culture was divided into two parts. The total RNA was isolated from the one and the crude cell extract was prepared from the other.

the respective species. In addition, we amplified the 5' untranscribed region of catalase genes of the three species and determined their sequences. Table 2 shows homology scores among catalases of known yeast species. The homology between the *C. albicans* and *C. dubliniensis* catalase is 99% in amino acid, indicating that the two species are closely related to each other. We observed a similar score (98%) in the topoisomerase II gene between the two species (Kanbe *et al.*, personal communication). On the other hand, homology between a *C. tropicalis* clinical isolate (NUM 5076) and *C. troplicalis* Pk233 was 95%, even though both strains are thought to belong to the same species[24]. Kato *et al.* suggests that the results of the score (99%) between *C. albicans* and *C. dubliniensis* is higher than that (95%) between *C. tropicalis* Pk233 and *C. tropicalis* NUM 5076 may reflect the difference in evolutionary path of the two phylogenetic clades[16].

The 5' untranscribed region of catalase genes of respective yeast species were amplified by PCR and their nucleotide sequences were determined. In spite of the homology between *C. albicans* and *C. dubliniensis* being not more than 70%, hypothetical motifs that may be involved in activation or repression of catalase gene expression such as STRE (stress responsive element) were conserved[6]. On the contrary, overall homology between *C. tropicalis* NUM 5076 and *C. tropicalis* Pk233 was not detected in the 5' untranscribed region, whereas the ORF region of the two catalases showed 95% homology in amino acids and 87% homology in nucleotides. We could not find any common sequence motifs in the 5' untranscribed region of the yeast catalase used in this study. In *S. cerevisiae*, there are two catalases in the genome, and they are distinguished from each other based on the regulation of transcription. All of the catalase genes examined so far in this study were transcribed, and catalase activities were successfully induced upon the treatment with a low concentration of hydrogen peroxide. This result may reflect that an unknown common mechanism is involved in transcriptional activation in spite of there being no obvious common sequence motif in the 5' untranscribed region among the yeasts.

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