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Expression of *Schizosaccharomyces pombe* Thioltransferase and Thioredoxin Genes under Limited Growth Conditions

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***Schizosaccharomyces pombe* gene encoding redox enzymes, such as thioltransferase (TTase) and thioredoxin (TRX), were previously cloned and induced by oxidative stress. In this investigation, their expressions were examined using β-galactosidase fusion plasmids. The expression of the two cloned genes appeared to be growth-dependent. The synthesis of β-galactosidase from the TTase-*lacZ* fusion was increased in the medium with the low glucose level, whereas it was significantly decreased in the medium without glucose or with galactose. It was also decreased in the nitrogen-limited medium. The synthesis of β-galactosidase from the TRX-*lacZ* fusion was unaffected by galactose or low glucose. However, it was lowered the absence of glucose. The synthesis of β-galactosidase from the TTase-*lacZ* fusion was shown to be enhanced in a higher medium pH. Our findings indicate that *S. pombe* TTase and TRX genes may be regulated by carbon and nitrogen sources, as well as medium pH.**

Keywords: Expression, Fission yeast, β-Galactosidase fusion, *Schizosaccharomyces pombe*, Thioltransferase, Thioredoxin

Introduction

Redox regulation is known to play an important role in diverse cellular functions (Powis *et al.*, 1995). The delicate interplay between oxidants and antioxidants ultimately determines the activity profile for various proteins, such as transcription factors. The activities and protein levels of antioxidant enzymes (such as superoxide dismutase, peroxidase, catalase, and glutathione *S*-transferase) are closely linked with the cellular responses to various oxidative stresses. Redox enzymes, such as thioltransferase (glutaredoxin) and thioredoxin, are also deeply involved in stress response.

Thioltransferase (TTase), also known as glutaredoxin, is a small, ubiquitous, multifunctional, glutathione-dependent disulfide oxidoreductase that reductively cleaves to a variety of disulfides, including protein disulfides and low-molecular-weight disulfides, by oxidizing reduced glutathione (Hatekeyama *et al.*, 1984). TTases that are purified from various organisms contain the active site sequence of -Cys-Pro-Phe(Tyr)-Cys-. The three-dimensional structure of a mammalian TTase was determined by a single crystal x-ray crystallography at 2.2 Å resolution (Katti *et al.*, 1995). It folds into an α/β structure with a four-stranded mixed β-sheet in the core, flanked on either side by helices. TTase participates in a pathway that couples the oxidation of NADPH to the reduction of ribonucleotide, sulfate, methionine sulfoxide, and arsenate. Some TTases are able to reduce non-disulfide substrates, such as dehydroascorbate and alloxan. Recently, TTase was found to play a central role in protection against protein damage that is caused by menadione and hydrogen peroxide in *Saccharomyces cerevisiae* (Rodriquez-Manzaneque *et al.*, 1999). Also, the expression of *S. cerevisiae* TTase genes was identified as induced in response to various stress conditions. These include oxidative, osmotic, and heat stress, as well as in response to the stationary phase growth and growth on non-fermentable carbon sources (Grant *et al.*, 2000). TTase (Zheng *et al.*, 1998) deactivates the OxyR transcription factor, which is activated through the formation of a disulfide bond. TTase, detected within HIV-1, is implicated as involved in the regulation and maintenance of protease activity in HIV-1 infected cells (Davis *et al.*, 1997). In v-jun transformed chicken embryo fibroblasts, the expression of TTase is a direct target of v-Jun (Goller *et al.*, 1998). TTase genes were isolated and their nucleotide sequences were determined from *Escherichia coli* (Höög *et al.*, 1986), the hyperthermophilic archaeon *Pyrococcus furious* (Guagliardi, 1995), and *S. cerevisiae* (Luikenhuis *et al.*, 1998). TTase cDNAs were obtained from mouse, pig, humans, and rice.

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Thioredoxin (TRX) is also a ubiquitous multifunctional protein having a redox-active disulfide/dithiol within its active site sequence, -Cys-Gly-Pro-Cys-, and operates together with NADPH and TRX reductase. TRX is a potent protein disulfide oxidoreductase that is important in the antioxidant defense (Nakamura *et al.*, 1997), regulation of cellular proliferation (Powis *et al.*, 1994), and regulation of the gene expression through transcription factor activation (Hayashi *et al.*, 1993). In addition, it is a powerful singlet oxygen quencher and hydroxyl radical scavenger (Das and Das, 2000). TRX negatively regulates p38 MAP kinase activation and IL-6 production by the tumor necrosis factor-α (Hashimoto *et al.*, 1999). TRX is known to regulate the growth rate of cells. It is also a critical component in the pathway leading to drug-induced apoptosis in mouse lymphocytic leukemia cells (Freemerman and Powis, 2000). The reduction activity of the TRX system could modify the antioxidant defenses of *Mycobacterium tuberculosis* (Zhang *et al.*, 1999). A redox-dependent function of TRX is necessary to sustain a rapid rate of DNA synthesis in *Saccharomyces cerevisiae* (Muller, 1995).

Previously, TTase and TRX genes of the fission yeast *Schizosaccharomyces pombe* were isolated and shown to be induced by oxidative stresses (Kim *et al.*, 1999; Cho *et al.*, 2000a,b,c; Cho *et al.*, 2001a,b). The characteristics of the growth and division of *S. pombe* cells are highly dependent upon the medium in which they are grown. Since thioltransferase and thioredoxin play important roles in the maintenance of the reduce states of the cells, their expressions are assumed to be closely linked with the medium condition. In this article, the expression of redox enzymes, such as TTase and TRX, was investigated under the limited growth conditions of *S. pombe*.

Material and Methods

Chemicals Bovine serum albumin (BSA), D-glucose, D-galactose, O-nitrophenyl β-D-galactopyranoside (ONPG), yeast glutathione reductase (GR), NADPH, Tris, reduced glutathione (GSH), leucine, uracil, adenine, and Bradford reagent were purchased from the Sigma Chemical Co. (St. Louis, USA). The 2-Hydroxyethyl disulfide (HED) was from the Aldrich Chemical Co. (Milwaukee, USA). All other chemicals and reagents used were of highest grade commercially available.

Strains and growth condition The *S. pombe* KP1 (*h⁺ leu-32 ura4-294*) (Cho *et al.*, 2001a; Kang *et al.*, 2001; Kim *et al.*, 2001) was used as a host for transformation. The yeast cells were grown in a minimal medium, which contained KH phthalate (3 g), Na_2HPO_4 (1.8 g), NH_4Cl (5 g), D-glucose (20 g), 1,000 × vitamin mixture (1 ml), 10,000 × minerals (0.1 ml), 50 × salts (20 ml), and L-leucine (250 mg) per 1 L. Construction of the *S. pombe* TTase-*lacZ* fusion plasmid was described previously (Cho *et al.*, 2000c). The *S. pombe* TRX-*lacZ* fusion plasmid, which was constructed previously (Cho *et al.*, 2001a), was used in this study.

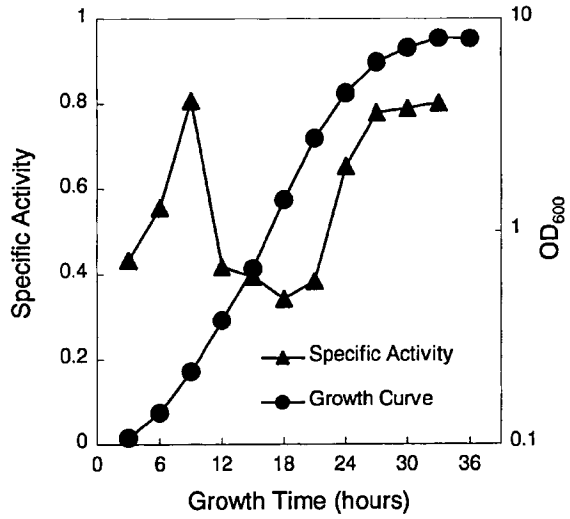

Fig. 1. The β-galactosidase activity (-▲-) of KP1/pYEHR1 was determined according to the growth curve (-●-). The *S. pombe* cells that harbor plasmid pYEHR1 were grown in a minimal medium with 250 mg/liter leucine at 30°C. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

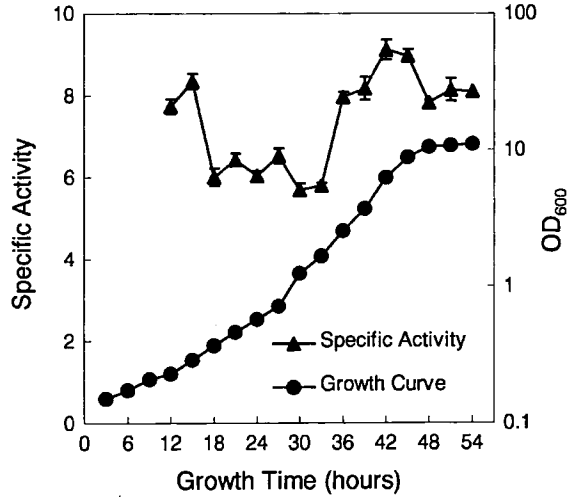

Fig. 2. The β-galactosidase activity (-▲-) of KP1/pYKT24 was determined according to the growth curve (-●-). The *S. pombe* cells that harbor plasmid pYKT24 were grown in a minimal medium with 250 mg/liter leucine at 30°C. The β-galactosidase activity was determined at 25°C by a spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

Cell harvest and disruption According to the time-course of shaking, the yeast cells were harvested by centrifugation. They were frozen and resuspended in 20 mM Tris-HCl buffer (pH 8.0) 2 mM EDTA (buffer A) and disrupted by using a glass bead beater and a sonicator. Supernatant was obtained after centrifugation and used as crude extract for enzyme assays (Kim *et al.*, 2001).

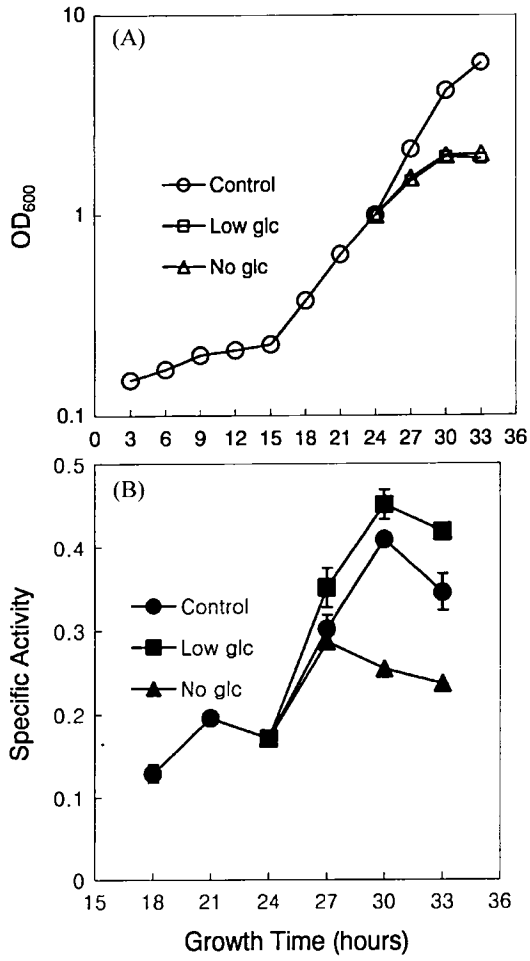

Fig. 3. The β-galactosidase activity of KP1/pYEHR1 was determined after a shift to limiting-glucose media. (A) Growth curve. (B) The β-galactosidase activity of KP1/pYEHR1. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

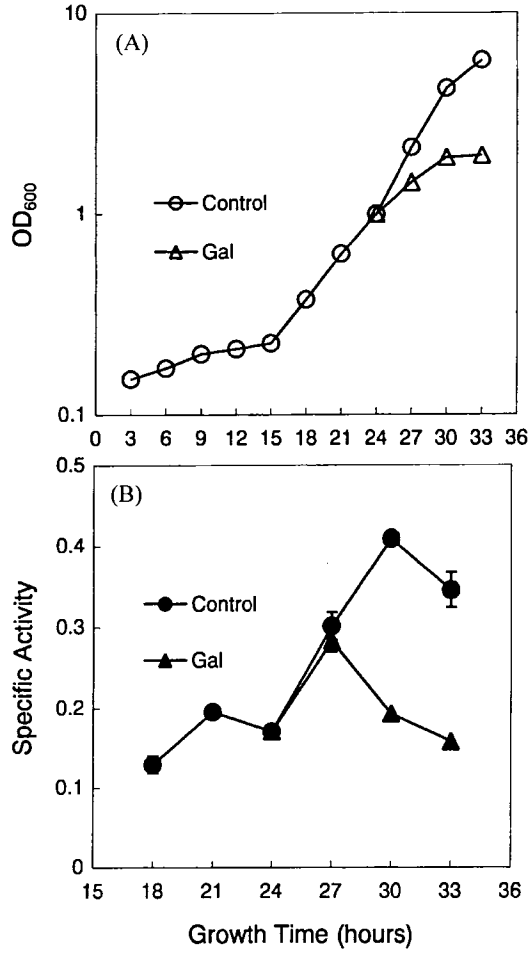

Fig. 4. The β-galactosidase activity of KP1/pYEHR1 was determined in the presence of galactose instead of glucose. (A) Growth curve. (B) The β-galactosidase activity of KP1/pYEHR1. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

β-Galactosidase assay β-Galactosidase activity in the extracts was measured by the spectrophotometric method (Guarente, 1983) using ONPG as a substrate. Protein contents in the extracts were measured by the Bradford method (Bradford, 1976) using BSA as a standard.

General techniques The other DNA techniques used in this study were performed according to Sambrook *et al.* (1989).

Results and Discussion

Growth-dependence Two redox enzymes, TTase and TRX, are believed to be involved in cellular responses to oxidative stresses. TRX activity is extensively required for rapidly growing mammalian cells (Grogan *et al.*, 2000). The expression of the two cloned genes that encode TTase and TRX of *S. pombe* was compared according to the growth cycle of the fission yeast cells. The shuttle plasmid pYEHR1 is a YEp357R derivative that harbors the 666 bp upstream sequence and the region encoding N-terminal 6 amino acids of the cloned TTase gene (Cho *et al.*, 2000c). It was constructed to independently monitor the expression of the cloned *S. pombe* TTase gene, since *S. pombe* was known to contain more than one TTase gene. As shown in Fig. 1, the synthesis of β-galactosidase from the TTase-*lacZ* fusion gave two peaks at the early exponential and early stationary phases. However, it showed a lower level of β-galactosidase at the mid-exponential phase (Fig. 1). These results indicate that the preservation of the reduced state is more important in the resting *S. pombe* cells. The shuttle plasmid pYKT24 contains *S. pombe* TRX-*lacZ* fusion, which harbors a 1270 bp upstream sequence of the cloned TRX gene (Cho *et al.*, 2001a). The *S. pombe* KP1, with plasmid pYKT24, appeared to give similar results with the same strain as pYEHR1 (Fig. 2). In the case of TRX-*lacZ* fusion, β-galactosidase synthesis varies in a relatively narrow range. These results suggest that

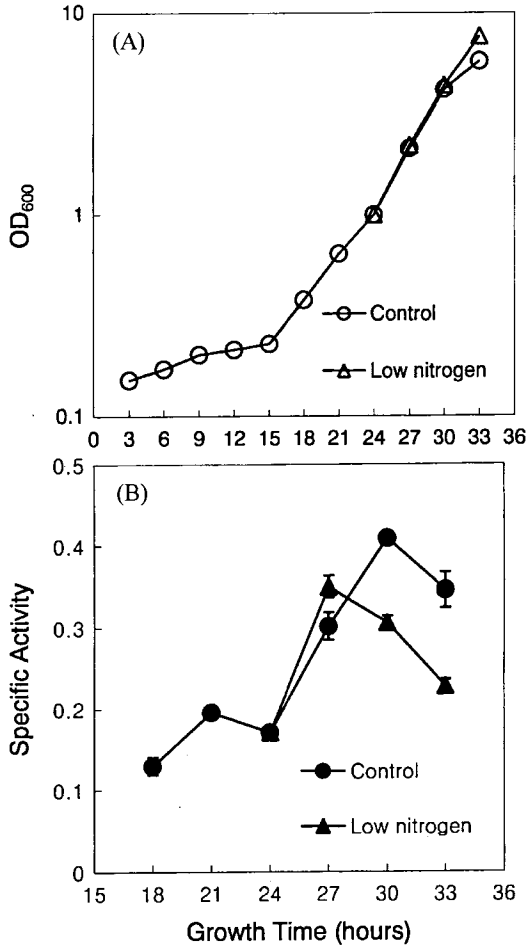

Fig. 5. The β-galactosidase activity of KP1/pYEHR1 was determined after a shift to a limiting-nitrogen source. (A) Growth curve. (B) The β-galactosidase activity of KP1/pYEHR1. The β-galactosidase activity was determined at 25°C by a spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

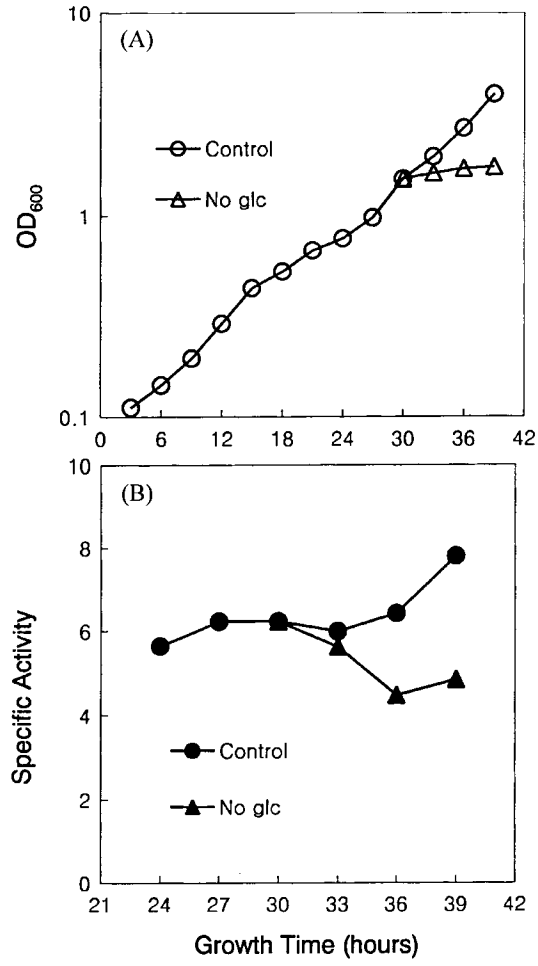

Fig. 6. The β-galactosidase activity of KP1/pYKT24 was determined after a shift to a limiting-glucose media. (A) Growth curve. (B) The β-galactosidase activity of KP1/pYKT24. The β-galactosidase activity was determined at 25°C by a spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

the TTase and TRX levels could be closely linked with the growth of the fission yeast. Growth-dependent regulatory mechanisms of *S. pombe* TTase and TRX genes remain to be elucidated.

Expression of TTase under limited conditions The *S. pombe* culture, containing the fusion plasmid pYEHR1, was grown in minimal medium at 30°C. A part of the culture at the exponential phase was transferred under limited growth conditions. Before the conditions were switched, the exponential cells were completely washed. In the glucose-deficient and low glucose (0.5%) media, the growth of the yeast cells was partly arrested (Fig. 3A). The synthesis of β-galactosidase from the fusion plasmid was slightly enhanced in the case of low glucose, whereas it was significantly decreased in the case of no glucose (Fig. 3B). This might suggest that the reduced state is easier to form in the growth-arrested cells. Although both of the two situations delayed the cell growth in a similar way, they showed an opposite pattern in the expression of the cloned TTase gene. The reduced expression of the TTase gene in the glucose-deficient condition could be interpreted by its low-level requirement in the delayed growth of cells. When the exponential cells were transferred into the minimal medium with galactose (2%), the growth of the yeast cells was delayed (Fig. 4A). According to the delayed growth, the synthesis of β-galactosidase from the fusion plasmid was markedly reduced (Fig. 4B). The reduced expression reflects its low-level requirement. The exponential cells were transferred into the low-nitrogen (5 mM NH_4Cl) media (Fig. 5A). Under the low-nitrogen condition, the growth of the cells was unchanged (Fig. 5A). However, the β-galactosidase synthesis from the fusion-plasmid was reduced (Fig. 5B). This could be related to the low level of protein biosynthesis. Taken together, the expression of the cloned *S. pombe* TTase gene was affected by carbon and nitrogen

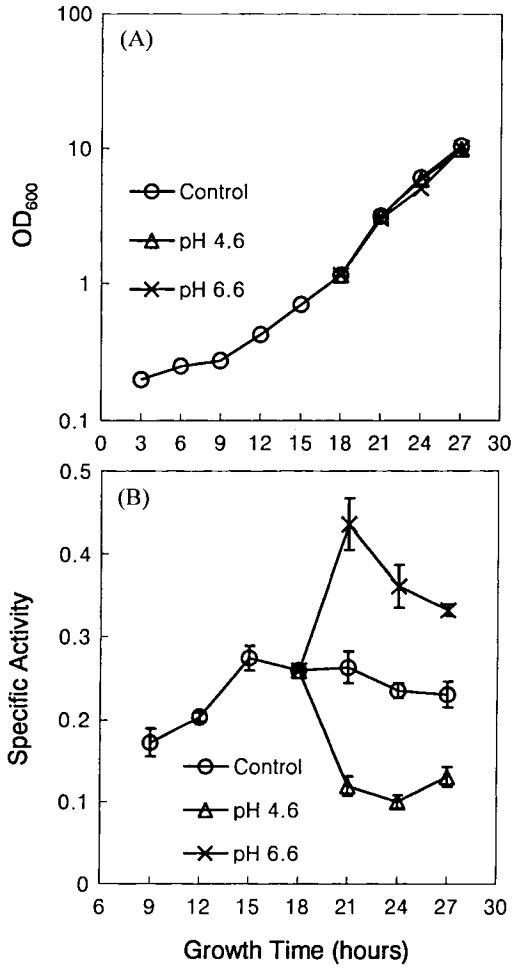

Fig. 7. The β-galactosidase activity of KP1/pYEHR1 was determined according to a change in pH. (A) Growth curve. (B) The β-galactosidase activity of KP1/pYEHR1. The β-galactosidase activity was determined at 25°C by a spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

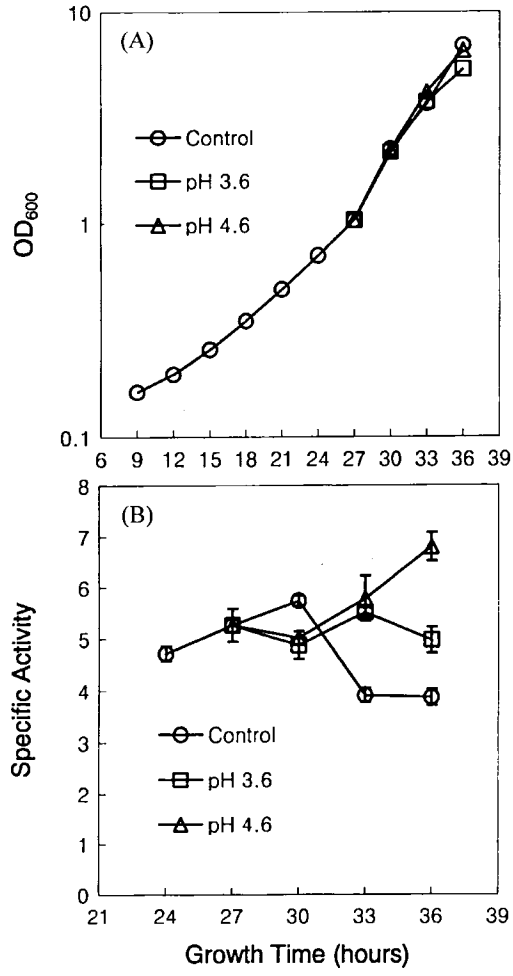

Fig. 8. The β-galactosidase activity of KP1/pYKT24 was determined according to a change in pH. (A) Growth curve. (B) The β-galactosidase activity of KP1/pYKT24. The β-galactosidase activity was determined at 25°C by a spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in μmol/min/mg protein.

sources, and their concentrations. This would confirm that TTase is involved in the growth rate of the yeast cells.

Expression of TRX under limited conditions The fission yeast cells that harbor the fusion plasmid pYKT24 were grown in a minimal medium at 30°C. Some of exponential cells were transferred under limited conditions. When the cells were transferred into low glucose, or low nitrogen growth conditions, the growth of the cells was strongly arrested (data not shown). However, the synthesis of β-galactosidase from the fusion plasmid was unchanged (data not shown). When the exponential cells were shifted into the no glucose medium, the growth of the cells was also arrested (Fig. 6A). The synthesis of β-galactosidase from the fusion plasmid pYKT24 was significantly decreased (Fig. 6B). These findings indicate that the regulation of the cloned TRX gene does not correspond with the TTase gene. Although the two redox systems share some functions, their regulations may be different.

pH-dependence The medium pH was assumed to affect the expression of the two redox enzymes in fission yeast. The pH of the standard minimal medium was approximately 5.6. Hydrochloric acid and sodium hydroxide were used for pH adjustment of the medium. For example, omitting Na_2HPO_4, adding 30 mM NaCl, and using H_3PO_4 for the pH adjustment accomplished the adjustment to pH 4.6. There, the phosphate did not limit growth under these conditions. When the *S. pombe* cells with pYEHR1 was transferred to the lower (pH 4.6) or higher (pH 6.6) pH of the medium, the growth rate did not vary (Fig. 7A). The synthesis of β-galactosidase from the fusion plasmid was significantly enhanced after the shift to pH 6.6, whereas it was decreased after the shift to pH 4.6 (Fig. 7B). Considering the pH 5.5 of the standard medium, the

expression of the TTase gene increased in the higher pH of the medium. When the yeast cells that harbor the fusion plasmid pYKT24 was transferred into the lower pH, the cell growth was unchanged (Fig. 8A). However, the synthesis of β-galactosidase from the fusion plasmid appeared to be slightly higher, although it was insignificant (Fig. 8B). There were also different patterns compared to that of the TTase gene.

Our results indicate that the expression of the redox enzymes, such as thioltransferase and thioredoxin, varies with the medium condition. However, the significance of this has not yet been precisely determined. The results from this investigation can be used as an important background for a mechanism study on the regulation of the two redox enzymes.

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