Evidence for the involvement of the *GTS1* gene product in the regulation of biological rhythms in the continuous culture of the yeast *Saccharomyces cerevisiae*

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Abstract In the yeast *Saccharomyces cerevisiae*, ultradian oscillations of energy metabolism have been observed in continuous cultures. Here, we found that the level of the *GTS1* gene product oscillated in concert with the ultradian rhythm of energy metabolism. When *GTS1* was inactivated by gene disruption, the metabolic oscillation was affected severely, mostly disappearing within a day, in the absence of synchronized stress-response oscillations throughout the continuous culture. The disappearance of biological rhythms in the *GTS1*-deleted mutant was substantially rescued by transformation with chimera plasmids carrying *GTS1* under the control of *GTS1*'s own promoter. On the other hand, this disappearance was not rescued by constitutive expression of *GTS1* under the control of the triose phosphate isomerase promoter.

Key words: Biological rhythm; Stress response; *GTS1*; Continuous culture; Yeast

1. Introduction

In the yeast *Saccharomyces cerevisiae*, sustained oscillations of energy metabolism have been observed in continuous cultures of the yeast [1–4]. These oscillations arise spontaneously under aerobic conditions in a manner dependent on high cell density when the dilution rate (the flow rate of medium (ml h^{-1}) through the culture vessel in proportion to the total volume of culture (ml)) and the physical parameters such as temperature, speed of agitation, aeration rate and so on are kept constant. The oscillations are detectable as a periodic change in respiratory–fermentative metabolism factors such as O_2 consumption, CO_2 production and glucose and ethanol concentrations [1–4]. The frequencies of the oscillation are dependent on the dilution rates [5] and not affected by temperature at least between 20 and 30°C (unpublished data). Keeping in mind what we knew about these oscillations, we found that cellular responses to various stress conditions such as heat, oxidative agents and other cytotoxic chemicals oscillated with the same frequency of the ultradian oscillations of metabolism [6].

We have reported that the gene *GTS1* isolated from the yeast *S. cerevisiae* [7,8] shows pleiotropic effects on yeast, including the timing of budding and sporulation and the capacity for heat tolerance in the stationary phase in a gene dose-dependent manner [7,9]. Either disruption or overexpression of *GTS1* affected the growth rate in the presence of cytotoxic drugs [10] and shortened yeast life spans [9]. Furthermore, *GTS1* affected the ultradian oscillation of the glycolytic metabolite NADH [11], which is induced by the addition of glucose followed by cyanide to a starved yeast (for review see [12]). The amplitudes and durations of the NADH oscillations were changed significantly as a function of the *GTS1* gene dosage [11].

In the present study, to examine whether the *GTS1* gene product, Gts1p, is involved in the regulation of biological rhythms in yeast, we determined whether the gene inactivation affects the appearance of various ultradian oscillation in the continuous culture.

2. Materials and methods

2.1. Yeast strain and culture conditions

The haploid strain S288C (*MAT*α *mal gal2 SUC2*) of *S. cerevisiae* was used. The cells were cultured at 30°C in a synthetic medium containing 1% glucose as defined elsewhere [3] using a bench-top fermenter, MDL-6C (Marubishi Bioengineering, Tokyo, Japan) with a constant volume of 500 ml [6]. Continuous cultures were started 2 h after the consumption of ethanol from the medium of batch culture and continued at the dilution of 0.1 h^{-1} with the same medium containing 1% glucose at pH 5.5. The periodic change of respiratory–fermentative metabolism was monitored by measuring the level of dissolved oxygen (DO) with an oxygen electrode. The amplitude of the wave was estimated by subtracting the DO concentration (%) of valley from that of the peak of the next wave.

2.2. Construction of GTS1 deletion mutant and transformation with a chimeric plasmid harboring GTS1

GTS1 deletion mutant of S288C was obtained using the *kan* gene as selective marker whose expression confers resistance to the antibiotic G418 in yeasts [13]. The 3.07 kbp *Sal*I–*Eco*RV fragment containing the *kan* gene was obtained from pNF4 [13] and inserted into the recombinant plasmid $pUCGTS_{RSP}$, which carries the 1.8 kbp *Eco*RI–*Spe*I fragment of *GTS1* [6] at the multi-cloning site of pUC119, replacing the 848 bp *Bam*HI–*Eco*RV fragment of *GTS1*. The resulting recombinant plasmid pDBVgtsNEO (*gts1::kan*) was digested with *Sal*I and *Eco*RI, and the purified *Sal*I–*Eco*RI fragment was transformed into S288C, yielding a deletion mutant of *GTS1*. Deletion of *GTS1* locus in S288C was confirmed by determining a physical map of the DNA fragment of the locus amplified by polymerase chain reaction (PCR).

To construct the centromeric plasmid pAUR112 (Takara, Tokyo, Japan) carrying *GTS1* with its own promoter, the 3.0 kbp *Sph*I–*Spe*I fragment, which was amplified by PCR directed on genomic DNA using synthetic oligonucleotides 5′-TATAGGAATTCATGGGTAACATTTTTAG-3′ and 5′-AATCTAAGCTTCTATAACTGTTCCTC-

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3′ as 5′ and 3′ primers, respectively, was inserted into pUC119 [7]. The *Nhe*I–*Pvu*II fragment obtained from the recombinant plasmid was inserted into the *Sal*I site of pAUR112 containing *CEN4* and *AUR1*. The resulted chimera plasmid pAC*GTS1*(NS) carrying the *GTS1* gene flanked by 1042 and 322 bp on the 5′ and 3′ sides, respectively, was transformed using the *GTS1*-deleted mutant of S288C. Transformant was selected on YPAD agar plates containing 2 μg ml^{-1} Aureobasidin A (Takara, Tokyo, Japan).

The multicopy plasmid YEp24 carrying *GTS1* with its own promoter was constructed by inserting the 1.3 kbp *Eco*RI–*Nco*I fragment, which was PCR-amplified using oligonucleotides 5′-CCCGAAGCATGCTGTGCTCAC-3′ and 5′-CTTAAAGCTTTTGTGTGTAGAAATAACC-3′ as 5′ and 3′ primers, into pCT119 [7]. The *Sal*I–*Spe*I fragment obtained from the recombinant plasmid was inserted into YEp24 and then the *Sph*I–*Nhe*I fragment was deleted. The resulted plasmid containing *GTS1* flanked by 1042 and 322 bp on the 5′ and 3′ sides, respectively, was inserted at the *Sal*I site with the *Sal*I–*Sac*I fragment containing *AUR1* from pAUR112. The recombinant plasmid pYE*GTS1*(NS) was transformed into the *GTS1*-deleted mutant of S288C.

To construct an expression vector carrying *GTS1* under the control of triose phosphate isomerase gene (*TPI*) promoter, the ORF of *GTS1* was PCR-amplified using synthetic primers 5′-ACTTAGGAATTCATGAGGTTTAGGAGTTCTTCC-3′ and 5′-ATCTTGAAGCTTTTGTGTGTAGAAATAACC-3′as 5′ and 3′ primers; these primers contain *Eco*RI and *Hin*dIII sites, respectively. The *Eco*RI–*Hin*dII fragment was then inserted into pYX222 vector (Takara, Tokyo, Japan) downstream of *TPI* promoter at *Eco*RI and *Hin*dIII sites. *AUR1* gene fragment, which was digested from the plasmid pAUR112, was inserted into this recombinant plasmid at *Sac*I and *Sal*I sites. Then, the obtained recombinant plasmid pYXTPIpr.-*GTS1* was transformed into the *GTS1*-deleted mutant of S288C.

2.3. Western blotting

Western blots were obtained as described previously using anti-Gts1p and anti-actin antibodies [7,10]. The relative protein level of Gts1p to actin was determined with a fluorometric bio-image analyzer (Takara FMBIO-100, Tokyo, Japan).

3. Results

3.1. Biological rhythms and fluctuation in Gts1p level of the wild-type yeast in the continuous culture

The DO oscillations observed in the continuous culture were caused by the periodic change from the respiratory to the respiro-fermentative phases of glucose metabolism, during which oxygen demand is relatively high and low, respectively [2,4]. When the dilution rate was fixed at 0.1, the level of DO oscillated with a period of about 4 h (Fig. 1A). The DO oscillation remained stable for more than 5 days. The metabolic oscillation had the same periodicity as that of the cycle of cell proliferation (Fig. 1B) as well as of the oscillations of

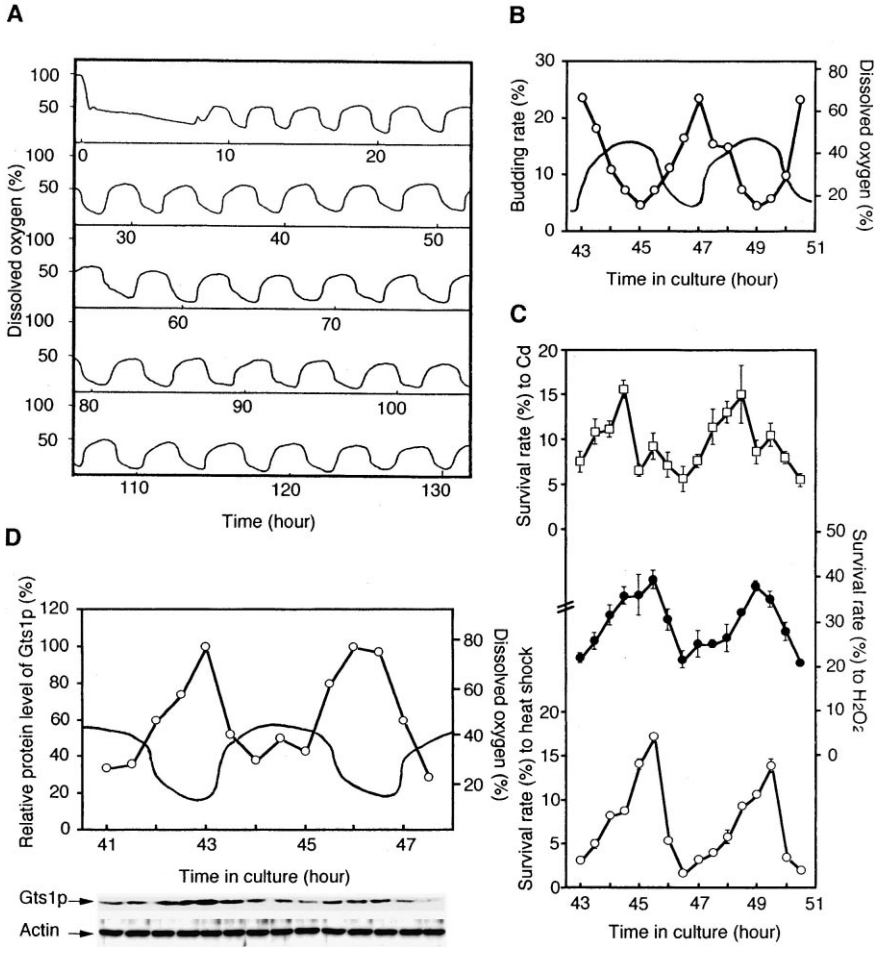

Fig. 1. Biological rhythms in a continuous culture of the wild-type yeast S288C. A: Oscillation of DO. Continuous culture was started at time zero and continued at the dilution of 0.1 h^{-1} with a synthetic medium containing 1% glucose at 30°C. B: Oscillation of budding rate (○) and (C) those of cellular resistance to heating at 55°C for 3 min (○), 3 mM H_2O_2 (●) and 20 mM cadmium (□). Solid line in (B) indicates DO. Cells were harvested at 30 min intervals from the effluent of the fermenter and assayed in triplicate. These are representative patterns of 10 independent experiments. D: Relative protein level of Gts1p to actin (○) determined by Western blotting. The Gts1p level was presented as percentage of the highest level. Solid line indicates DO.

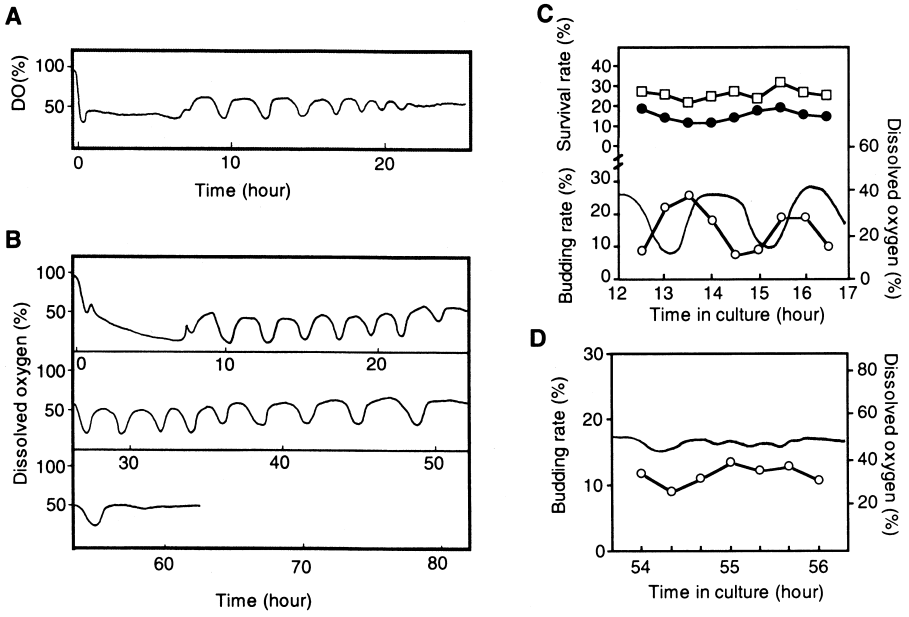

Fig. 2. Oscillations of biological rhythms in a continuous culture of *GTS1*-deleted mutant. A: The most representative pattern in which the DO oscillation ceased within a day. B: The pattern of the longest oscillation among the experiments. C, D: Patterns of budding rate (○) and cellular resistance to heating at 55°C for 3 min (●) and 20 mM cadmium (□) during the early (C) and late (D) times of the longest metabolic oscillation in (B). Cells were harvested at (C) 30 min or (D) 20 min intervals from the effluent of the fermenter and assayed in triplicate. Solid line indicates DO.

cellular responses to a variety of stresses, such as heat, H_2O_2 and the cytotoxic metal cadmium (Fig. 1C), in agreement with previous results [6]. The Gts1p level also oscillated in tandem with the energy metabolism oscillation, peaking at the respiratory phase (Fig. 1D).

3.2. *Effect of GTS1-inactivation on the biological rhythms in yeast*

When a *GTS1*-deleted mutant was applied to the continuous culture, the biological rhythms were greatly affected, although the degree of oscillation varied among the different experiments. Each of the seven experiments used a different batch of the *GTS1*-deleted mutant. Of these, two did not exhibit any oscillations (data not shown) and four started oscillating, but these oscillations faded within 24 h (Fig. 2A). In the last case of the seven cases, the metabolic oscillation continued for as long as 2 days (Fig. 2B), but the oscillation was much more irregular in both amplitude and periodicity than it was in the wild-type cells (Table 1). Stress-response oscillations were not found at any time during the metabolic oscillation while the cell division cycle was found (Fig. 2C,D). These results suggest that *GTS1* is required for the appearance of biological rhythms and that the cell cycle rhythm is more strongly connected to the oscillation of energy metabolism than to the oscillation of stress responses in the absence of Gts1p.

3.3. *Rescue of GTS1 inactivation by expression of the gene under the control of its own and heterologous promoters*

To examine whether or not expression of the *GTS1* gene under the control of its own promoter can rescue the disappearance of biological rhythms in the *GTS1*-deleted mutant, the centromeric recombinant plasmid carrying *GTS1* with its upstream region of about 1.0 kbp (pAC*GTS1*(NS)) was transformed. The energy metabolism oscillation continued for about 4 days in all four experiments (Fig. 3A), although the amplitude of DO oscillation was about 30% lower than it was in the wild-type cell (Table 1). The cell division cycle (Fig. 3B)

Table 1
Wavelengths and amplitudes of the DO oscillations in the wild-type and *GTS1*-deleted mutant with and without transformation with pAC*GTS1*(NS) or pXY*GTS1*(NS)

Strains	No of wave[c] (waves/run)	Wavelength[c] (h/wave)	Amplitude[d] (% DO)
Wild-type	34 ± 1.53 (n = 5)[a]	3.90 ± 0.34 (100)	40.4 ± 2.21 (100)
TMΔgts1	15 (n = 1)[b]	2.72 ± 0.80 (70)	27.0 ± 3.12 (67)
TMΔgts1 transformed with:			
pAC*GTS1*(NS)	29 ± 2.38 (n = 3)[a]	4.04 ± 0.30 (104)	28.4 ± 2.89 (70)
pXY*GTS1*(NS)	20 ± 2.83 (n = 2)[a]	3.80 ± 0.54 (97)	33.0 ± 6.80 (83)

Numbers in parentheses indicate percent of control.
[a]The number of experiments used for statistics. The number is less than that described in the text because some experiments were stopped before the disappearance of DO oscillations.
[b]The experiment of the longest oscillation shown in Fig. 2B.
[c]Average and standard deviation of the number of waves in one oscillation and the wavelength determined with all experimental values for each strain.
[d]Average and standard deviation of the amplitude of all waves for each strain estimated by subtracting the DO concentration (%) of the valley from that of the peak of the next wave.

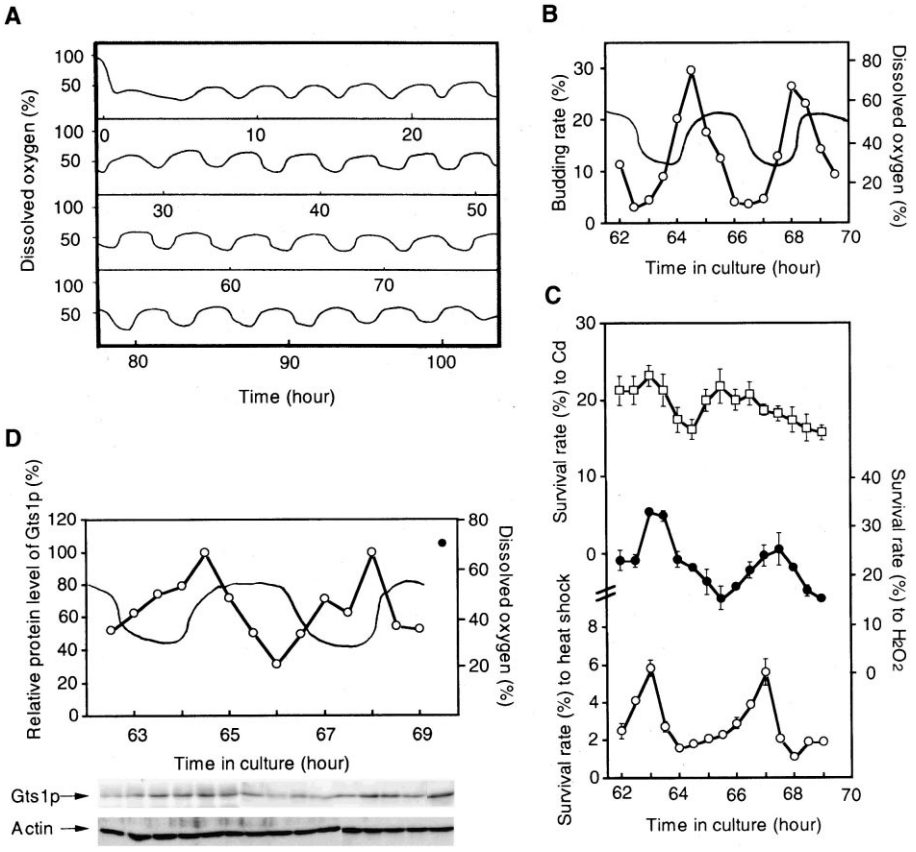

Fig. 3. Biological rhythms in a continuous culture of *GTS1*-deleted mutant transformed with a chimera plasmid harboring *GTS1* under the control of its own promoter. A: Oscillation of DO. This is a representative pattern of four independent experiments. B and C: Patterns of budding rate (○) in (B), and cellular resistance to heating at 55°C for 3 min (○), 3 mM H_2O_2 (●) and 20 mM cadmium (□) in (C). Cells were assayed in triplicate. D: Relative protein level of Gts1p to actin (○) during the DO oscillation (solid line). The Gts1p level was presented as percentage of the highest level. The relative Gts1p level at peak of the DO oscillation in the wild-type strain as a control (●).

and cellular resistances to heat and hydrogen peroxide were well oscillated, whereas the resistance to cadmium was weakly oscillated (Fig. 3C). Gts1p oscillated at a level nearly identical to that of the wild-type cell, judging from the relative protein level of Gts1p to actin. These results suggested that expression of *GTS1* could rescue, if not perfect, the disappearance of biological rhythms in the *GTS1*-deleted mutant.

To examine whether or not the rhythmic expression of Gts1p is required for the appearance of the metabolic oscillation, *GTS1* was expressed under the control of the *TPI* promoter in a constitutive manner [14]. When the recombinant plasmid pYXTPIpr.-*GTS1* was expressed in the *GTS1*-deleted mutant, the DO oscillation was unstable and irregular in all five experiments and mostly faded within 24 h (Fig. 4A). The Gts1p level, which was several times higher than that of the wild-type cell, did not fluctuate during the continuous culture (data not shown). Thus, to rule out the possibility that the protein level is more important than rhythmic expression of Gts1p for the appearance of biological rhythms, the multicopy recombinant plasmid carrying *GTS1* with its upstream region of about 1.0 kbp (pYE*GTS1*(NS)) was used. When the transformant was applied to the continuous culture, the DO oscillation continued for longer than 3 days (Fig. 4B and Table 1). The cell division cycle (data not shown) and cellular resistances to heat and hydrogen peroxide were well oscillated, whereas the resistance to cadmium was weakly oscillated (Fig. 4C), similar to what we saw using the transformant with the centromeric pAC*GTS1*(NS) (Fig. 3C). The Gts1p level was about four times the level in the wild-type cell, and the amplitude (ratio of peak protein level to valley protein level = 2.0) was about 40% shallower than it was in the wild-type cell (peak/valley ratio = 3.3) (Fig. 4D).

4. Discussion

In this communication, we showed that the Gts1p level oscillated whenever the biological rhythms appeared in the continuous culture. When *GTS1* was expressed in a rhythmic fashion under the control of its own promoter in either a centromeric or a multicopy plasmids, the biological rhythms continued for more than 3 days. On the other hand, when *GTS1* was inactivated or constitutively expressed under the control of the *TPI* promoter, the biological rhythms disappeared mostly within a day. Thus, it is likely that the rhythmic expression of Gts1p is more important than the protein level for the maintenance of the biological rhythms. However, an adequate level of Gts1p seems to be required for the stable oscillation of the biological rhythms, because the DO oscillation lasted longer with relatively constant amplitudes in the transformant with the centromeric, as opposed to the multicopy, plasmid. (Table 1). It is worth noting that the wavelength of the DO oscillation was not affected in these transformants (Table 1). This is probably because the wavelength of the metabolic oscillation depends on the dilution rate [5] –

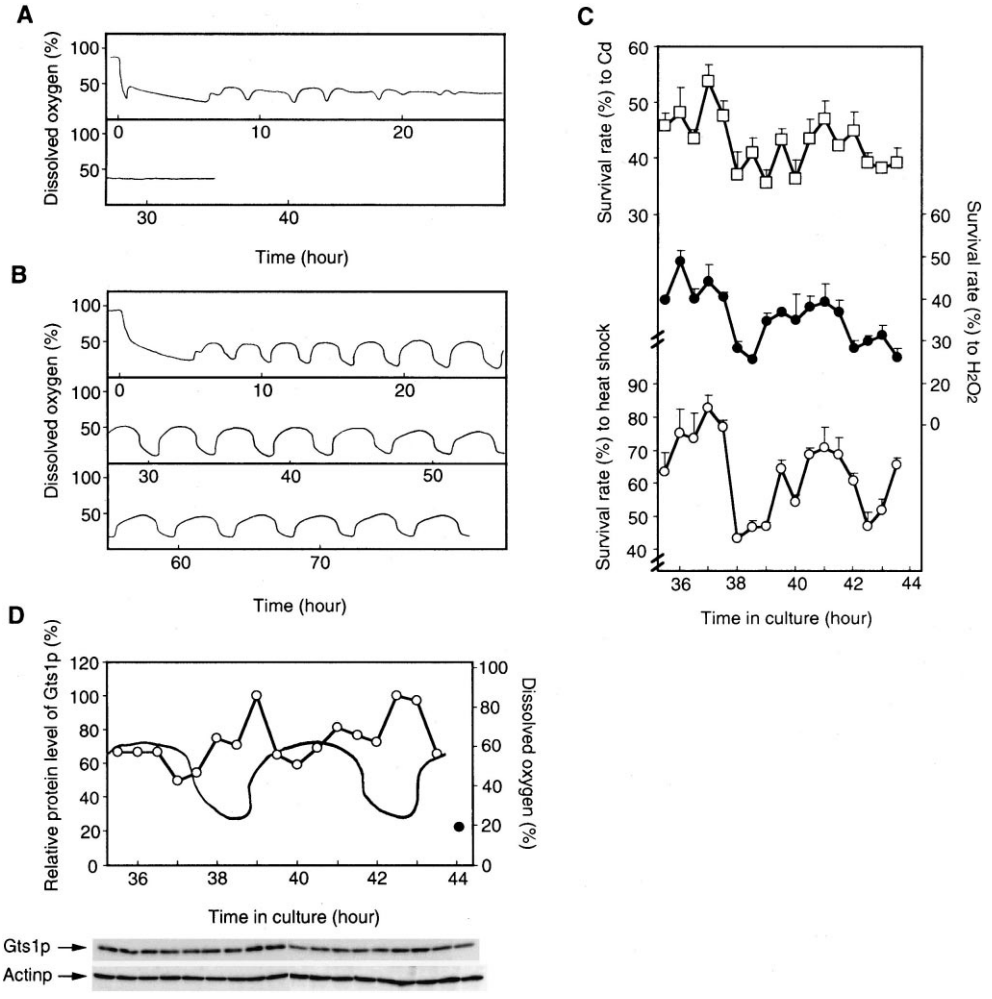

Fig. 4. Oscillations of biological rhythms in transformants with multicopy numbers of *GTS1* under the control of *TPI* (A) and its own promoters (B–D). A: The representative pattern of the DO oscillation in a continuous culture of *GTS1*-deleted mutant transformed with pYXT-PIpr.-*GTS1*. B: The representative pattern of the DO oscillation in a continuous culture of *GTS1*-deleted mutant transformed with pYE*GTS1*(NS). C: Pattern of cellular resistance to heating at 55°C for 3 min (○), 3 mM H_2O_2 (●) and 20 mM cadmium (□) assayed in triplicate. D: Relative protein level of Gts1p to actin (○) during the DO oscillation (solid line). The Gts1p level was presented as percentage of the highest level. The relative Gts1p level at the peak of the DO oscillation in the wild-type strain was used as a control (●).

that is, the inflow rate of glucose as a sole energy source – and because the ultradian rhythm is unnecessary to adapt to a particular environmental periodicity such as the day–night cycle.

Glycolytic oscillations have been proposed to occur autogenously under the primary control of phosphofructokinase, which is autocatalytically activated by its own product ADP [12,15]. The energy metabolism pathway has been proved to be a sustained oscillator under extreme non-equilibrium conditions of energy according to the theory of 'dissipative structures' established by Prigogine and co-investigators [16–18]. The metabolic pathway oscillates autogenously, transferring energy from glucose to NADH, which acts as the feed-forward activator, and then from NADH to ATP, which acts as the feedback inhibitor. Thus, it is possible that, in yeast, the metabolic oscillator drives other oscillators by supplying the final metabolite ATP in a non-linear fashion, while the precise molecular mechanism, including the function of Gts1p, remains to be clarified.

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