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Application of a phosphite dehydrogenase gene as a novel dominant selection marker for yeasts

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ABSTRACT

The use of antibiotic resistance markers in the commercial application of genetically modified microorganisms is limited due to restrictions on the release of antibiotics and their resistance genes to the environment. To avoid contamination by other microorganisms, the development of a dominant selection marker with low environmental risks is still needed. Here we demonstrated a new selection system for *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* using a bacterial phosphite dehydrogenase gene (*ptxD*). A *Sz. pombe* transformant carrying *ptxD* under a strong promoter or on a multicopy plasmid grew on a minimal medium containing phosphite (P_t) as a sole source of phosphorus. To adapt this system to *S. cerevisiae* strains, codon optimization of *ptxD* was necessary. The codon-optimized *ptxD* system appeared effective in not only laboratorial but also industrial *S. cerevisiae* strains that are diploid or polyploid. Since P_t is a safe and inexpensive chemical, *ptxD* could be used as a novel dominant selection marker applicable on an industrial scale.

1. Introduction

Genetically modified microorganisms play a vital role in chemical, pharmaceutical and food industries. Selectable gene markers, which confer the ability to grow on a selective medium, are not only required for the transformation process but are also usually used to make the genetic phenotype dominate and to avoid contamination by other microorganisms in the subsequent culturing process. Antibiotics and their resistance genes have been widely used for this purpose in laboratory-scale experiments. However, their industrial uses are costly and have to be strictly regulated from an environmental health perspective since the release of antibiotics and genetically modified microorganisms carrying antibiotic resistance genes increases the risk of emergence of new drug-resistant microorganisms (Droge et al., 1998; Heinemann, 1999; Zhang et al., 2009).

The auxotrophic markers, which complement a host cell mutation in an essential metabolic pathway, have been widely used in yeasts (Buckholz and Gleeson, 1991; Hartley et al., 1980; Lin Cereghino et al., 2001). However, the isolation of recessive mutants is laborious and time-consuming, and sometimes results in the changes in the original phenotype due to the introduction of unwanted mutations into the genes other than the desired locus. These problems become particularly pronounced in industrial yeasts because their genomes are usually diploid or polyploid (Bidenne et al., 1992; Cebollero and Gonzalez, 2004). The commercial use of yeasts has expanded from food industry to the production of medicinal compounds, chemicals, and renewable biofuels (Nielsen, 2013). Therefore, the development of a dominant selection marker that allows economically viable yeast cultivation with low environmental risks should contribute to a number of industries.

Phosphorus is an essential nutrient for all living organisms. On earth virtually all known phosphorus exists in the +5 oxidation state. Phosphite (P_t) is a salt of phosphorous acid where phosphorus has an oxidation state of +3. P_t is a structural analogue of phosphate (P_i) that is efficiently absorbed by the P_i transport system. Since P_t inhibits the growth of oomycetes of the genus *Phytophthora*, a pathogen of plants, and activates plant defense mechanisms (Förster et al., 1998; Saindrenan et al., 1988), it is now increasingly used as alternative fungicides (Lobato et al., 2008; Mayton et al.,

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Table 1
Strains used in this study.

Strain or plasmid	Description	Reference or source
Sz. pombe strains		
L972	*h*⁻, prototroph	Our stock
L975	*h*⁺, prototroph	Our stock
KSP632	*h*⁻ *ura4-D18*	Kitamura et al. (2012)
KSP635	*h*⁻ *leu1-32*	Kitamura et al. (2012)
HFF1ptxD-g	*h*⁻, *leu1*⁺-[P*nmt1-ptxD*]int	This study
HFF41ptxD-g	*h*⁻, *leu1*⁺-[P*nmt41-ptxD*]int	This study
HFF81ptxD-g	*h*⁻, *leu1*⁺-[P*nmt81-ptxD*]int	This study
HFF1ptxD-p	*h*⁻, *ura4-D18* [pDUAL(P*nmt1-ptxD*)-*ura4*⁺]multicopy	This study
HFF41ptxD-p	*h*⁻, *ura4-D18* [pDUAL(P*nmt41-ptxD*)-*ura4*⁺]multicopy	This study
HFF81ptxD-p	*h*⁻, *ura4-D18* [pDUAL(P*nmt81-ptxD*)-*ura4*⁺]multicopy	This study
S. cerevisiae strains		
W303a	*MAT*a *ura3 his3 leu2 trp1 ade2 can1*, haploid	Our stock
Kyokai no. 6	Sake yeast, prototroph, diploid	NRIB
Kyokai no. 7	Sake yeast, prototroph, diploid	NRIB
Kyokai no. 9	Sake yeast, prototroph, diploid	NRIB
Shochu yeast SH-4	Shochu yeast, prototroph, diploid	NRIB

NRIB, National Research Institute of Brewing.

2008). US Environmental Protection Agency has classified P_t as a biopesticide whose use will not pose unreasonable risks of harm to human health and the environment (Lobato et al., 2008; Mayton et al., 2008). On the other hand, P_t must be oxidized to P_i prior to assimilation as a phosphorus source. P_t dehydrogenase (PtxD), catalyzing oxidation of P_t to P_i with the concomitant reduction of NAD^+ to NADH, has been found in several bacteria but not in eukaryotes (Costas et al., 2001; Vrtis et al., 2002). McDonald et al. (2001) has reported that P_t can be incorporated into *Saccharomyces cerevisiae* but not assimilated as a phosphorus source. Therefore, *ptxD* could become a candidate for a dominant selection marker in yeasts.

In this study, we first demonstrated that the introduction of *ptxD* to a fission yeast *Schizosaccharomyces pombe* and a budding yeast *S. cerevisiae*, both of which are important organisms for research and industrial purposes, confers the ability to grow on a P_t medium. Moreover, since P_t is an environmentally safe and inexpensive chemical compared to antibiotics, this selection system would offer a cost-effective method for large-scale cultivation of yeast recombinants with low environmental risk.

2. Materials and methods

2.1. Yeast strains and cell growth

Yeast strains used in this study are listed in Table 1. A complete YPD medium (1.0% yeast extract, 2.0% peptone, 2% dextrose) was used for routine culture of yeast strains. We prepared EM medium as a phosphorus-free defined minimum medium for *Sz. pombe* by removing P_i from components of a Edinburgh minimal medium (Forsburg and Rhind, 2006), and also prepared SD medium (0.58% yeast nitrogen base without amino acid and without phosphate purchased from Formedium [Norfolk, United Kingdom], 2% dextrose) as a phosphorus-free defined minimum medium for *S. cerevisiae*. Filter sterilized P_i or P_t (Nakalai Tesque, Kyoto, Japan) neutralized to pH 7.0 with NaOH was added to the EM and SD medium, the resultant media being designated as EM-P_i or EM-P_t and SD-P_i or SD-P_t, respectively. Phosphorus concentrations added to the EM and SD media were 15 mM and 7.5 mM, respectively. The prepared P_t solution contained trace amounts of P_i (approximately <0.3%), which gave a final concentration of less than 50 μM in the media. However, this amount was insufficient to support yeast growth (data not shown). A purchased agar also contained a trace amount of P_i. Prior to use, P_i was removed by washing three times with ultrapure deionized water. Amino acids and nucleotide bases were added as auxotrophic nutrients when necessary.

2.2. PtxD expression in Sz. pombe

A plasmid pDUAL was used not only for chromosomal integration of *ptxD* but also for episomal multicopy maintenance in *Sz. pombe* (Matsuyama et al., 2004). Three derivative plasmids pDUAL-HFF1, pDUAL-HFF41, and pDUAL-HFF81, regulated by *nmt1* promoter (P*nmt1*) and its two attenuated versions P*nmt41* and P*nmt81* (Basi et al., 1993) respectively, were used to analyze effect of levels of *ptxD* expression on yeast growth on the EM-P_t medium. A *ptxD* gene of *Ralstonia* sp. (Hirota et al., 2012) amplified using primer pairs (5′-CACCATCATCATATGAAGCCCAAAGTCGTCCTCAC-3′, 5′-ATCATCCTTATAATCTCACGCCGCCTTTACT CCCG-3′), and pDUAL-HFF1 DNA amplified using primer pairs (5′-CATATGATGATGGTGGTGATGCATAG-3′, 5′-GATTATAAGGATGATGA CGATAAAC-3′) were used to construct pSZPT1 (Fig. 1a) by In-Fusion cloning kit (Takara, Japan). The *ptxD* gene was inserted into downstream of *nmt1* promoter of pDUAL-HFF1. Plasmids pSZPT41 and pSZPT81 (Fig. 1a) were also constructed using pDUAL-HFF41 and pDUAL-HFF81, respectively.

2.3. PtxD expression in S. cerevisiae

To enhance the protein expression of *ptxD* in *S. cerevisiae*, arginine codons (CGC, CGA, CGG) and an alanine codon (GCG) of *ptxD* were changed to preferred codons in *S. cerevisiae* (AGA and GCT, respectively). The codon-optimized *ptxD* variant (OPT*ptxD*) was chemically synthesized by GenScript USA (Piscataway, NJ), and then inserted in between a promoter (P*ipc*) and a terminator (T*ipc*) of inositol phosphorylceramide (IPC) synthase gene (Hashida-Okado et al., 1996). The OPT*ptxD* gene, together with P*ipc* and T*ipc*, was amplified using a set of primers (5′-AGTCAGGCACCGTGTATAGCGTAAGAACACTAGCGAC-3′, 5′-GCCGCCGGCTTCCATAGAATAACGCAAAACCACCC-3′), and then replaced with a *tet* gene of YEp24 (Botstein et al., 1979) by In-Fusion cloning kit, the resultant plasmid being designated as pSCPT (Fig. 1b).

2.4. Transformation of yeast

A high-efficiency protocol (Matsuyama et al., 2000) was used for transforming *Sz. pombe* and *S. cerevisiae*. Briefly, exponentially growing cells in 3 ml of YPD medium were collected by centrifugation, washed once with 3 ml of sterilized water, and resuspended in 0.1 ml of lithium acetate (pH 5.0). Five micrograms of plasmid