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Soluble fumarate reductase isoenzymes from *Saccharomyces cerevisiae* are required for anaerobic growth

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Abstract

In *Saccharomyces cerevisiae*, the cytosolic and promitochondrial isoenzymes of fumarate reductase are encoded by the *FRDS* and *OSM1* genes, respectively. The product of the *OSM1* gene is reported to be required for growth in hypertonic medium. Simultaneous disruption of the *FRDS* and *OSM1* genes resulted in the inability of the yeasts to grow anaerobically on glucose as a carbon source, and disruption of the *OSM1* gene caused poor growth under anaerobic conditions. However, the disruption of both the *FRDS* and/or *OSM1* genes had no effect on aerobic growth or growth under hypertonic conditions. These results suggest that the fumarate reductase isoenzymes in *Saccharomyces cerevisiae* are essential for anaerobic growth but not for growth under hypertonic conditions. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Fumarate reductase; *OSM1* gene; Promitochondrial enzyme; Yeast

1. Introduction

The fumarate reductase enzyme of yeasts irreversibly catalyzes the reduction of fumarate to succinate [1]. The enzyme is a soluble protein, bound non-covalently to flavin adenine dinucleotide (FAD). In contrast, the fumarate reductase enzyme of *Escherichia coli* [2] and *Vibrio succinogenes* [3] are membrane-bound enzymes bound covalently with FAD, that exhibit weak succinate oxidation activity. Yeasts contain two fumarate reductase isoenzymes that can be fractionated into the absorbed (FRDS1; fumarate reductase soluble 1) and non-absorbed (FRDS2; fumarate reductase soluble 2) fractions on a DE-52 column [4]. Recently, we determined that FRDS1, located in the cytosol, is encoded by the *FRDS* gene [5] and FRDS2, located in promitochondria, is encoded by the *OSM1* gene [6]. Deletion of the *OSM1* gene has been reported to increase the sensitivity of the yeasts to hypertonic stress [7]. However, the metabolic roles of FRDS1 and FRDS2 are still not clear. We report here that disruption of the two fumarate reductase genes *FRDS* and *OSM1* results in inability of the yeasts to grow under anaerobic conditions, indicating that these enzymes are required for anaerobiosis.

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2. Materials and methods

2.1. Strains and plasmids

E. coli, strain XL1-Blue, was obtained from Stratagene (Heidelberg, Germany). *S. cerevisiae*, strain DURA (*MATa his3-Δ leu2-3 leu2-112 trp1-289a URA3*), which was obtained by integrating the *URA3* gene into strain DBY747 [8], was used for the growth experiments. *E. coli* plasmid vector pHSG398 [9] was purchased from Takara Shuzo Co. Plasmids pFRDS and pOSM1, bearing the *FRDS* and *OSM1* genes, respectively, were constructed during previous studies [5,6]. Two plasmids, pURAKm, which contained the *URA3* gene and the kanamycin resistant (Km^r) gene from pNH-Kan/*ori*T plasmid [10], and pHTHAKm, which contained β-thienyl alanine resistant (THA^r) [11] gene and the Km^r gene, were constructed and used for construction of the disruption cassettes of the *FRDS* and *OSM1* genes (see Fig. 1 and its legend). The Km^r gene is useful for the selection of a clone harboring a gene disruption plasmid in *E. coli*, and *URA3* and THA^r for the selection of a gene-disruptant in *S. cerevisiae*. A yeast gene library [11] was used for cloning the genes. The library was prepared by ligation of partial *Alu*I fragments (5–10 kilobase pairs (kb)) of the nuclear DNA of THA^r mutant strain of *S. cerevisiae*, K901-T12, to the *Bam*HI site of an episomal-type shuttle vector containing *LEU2* gene, and used for the cloning of the THA^r gene in our previous study [11].

2.2. Media

Yeast strains were grown in a nutrient medium composed of 2% peptone and 1% yeast extract supplemented with either 2% glucose (YPD), 10% glucose (YPD10) or 27% glucose (YPD27). For anaerobic cell culture, 6 ml of ergosterol solution (2 mg ml^{-1}) prepared as described previously [4], was added to 1 l of the above media. A synthetic glucose medium (SD) contained 0.67% yeast nitrogen base w/o amino acids (Difco), 2% glucose and appropriate amounts of required amino acids. To prepare agar plates, 2% agar was added to the media. *E. coli* strains were grown in 2YT broth (1.6% Trypton, 1% yeast extract, 0.5% NaCl, 0.1 mg ml^{-1} of thiamin) supplemented with 0.1 mg ml^{-1} ampicillin and/or 0.025 mg ml^{-1} kanamycin sulfate for selection of clone harboring the plasmid. For selection of β-thienyl alanine (THA)-resistant yeast transformants, 0.02 mg ml^{-1} THA (Sigma) was added to the SD plate.

2.3. DNA manipulation and transformation

DNA manipulations were carried out using conventional techniques [12]. The methods of Hanahan [13] and Ito et al. [14] were used respectively for the transformation of *E. coli* and *S. cerevisiae*. The one-step gene replacement in *S. cerevisiae* was carried out according to the method of Rothstein [15]. Southern blotting hybridization was carried out using random primer labeling and the signal amplification system for a fluorimager (Amersham) and a fluorodetector, Storm (Molecular Dynamics).

2.4. Growth of yeast

To study the growth of yeasts under aerobic conditions, L-shaped tubes (12 ml) containing 5 ml of the medium that were inoculated with fresh stationary-phase cells were incubated at 30°C with reciprocal shaking at 40 rpm. Growth was monitored by measuring OD_{660nm} using a biophotorecorder TN-1506 (Advantec, Japan). For anaerobic growth, tubes (30 ml) containing 20 ml of medium that were inoculated with cells and overlaid with 1.5 ml of liquid paraffin (a depth of 1 cm) were allowed to stand at 30°C. At prespecified times, the cultures were gently stirred, and the absorbance of the cultures was measured at 660 nm.

2.5. Assay of enzyme activity in cell-free extracts

Yeast cells were grown in a 500-ml Sakaguchi flask containing 400 ml of YPD10 medium for 30 h at 30°C with gentle shaking. The cells were suspended in 4 volumes of 20 mM K,Na-phosphate buffer (pH 7.0) containing 2 mM EDTA·2Na and 5 mM mercaptoethanol. The cells were disrupted by passing them twice through a French pressure cell (Ohtaki, Japan) at 2000 kg cm^{-2}. The cell-disrupted suspensions were centrifuged at $39\,000 \times g$ for 20 min. Fumarate reductase activity in the supernatant was determined according to a previously reported method

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[4]. A unit of activity was expressed as µmol of $FMNH_2$ oxidized per minute. Protein content was assayed by the method of Lowry et al. [16].

3. Results and discussion

3.1. Disruption of the FRDS and OSM1 genes encoding fumarate reductase

Approximately 50 uracil-independent clones were obtained by transforming DBY747 with the *FRDS::URA3* construct derived from plasmid pFRDU2 (Fig. 1) by cleavage with *Eco*RI. Similarly, the same yeast strain was transformed to Ura^+ phenotype with the *OSM1::URA3* construct derived from plasmid pOSMU1 (Fig. 1) by cleavage with *Bam*HI and *Cla*I.

Five clones selected at random were examined for the presence of the expected mutant allele by Southern blotting against their genomic DNAs (Fig. 2). Nuclear DNAs obtained from the Ura^+ or THA^r transformants and the wild-type parent strain DBY747 were digested with *Hin*dIII, size-fraction-

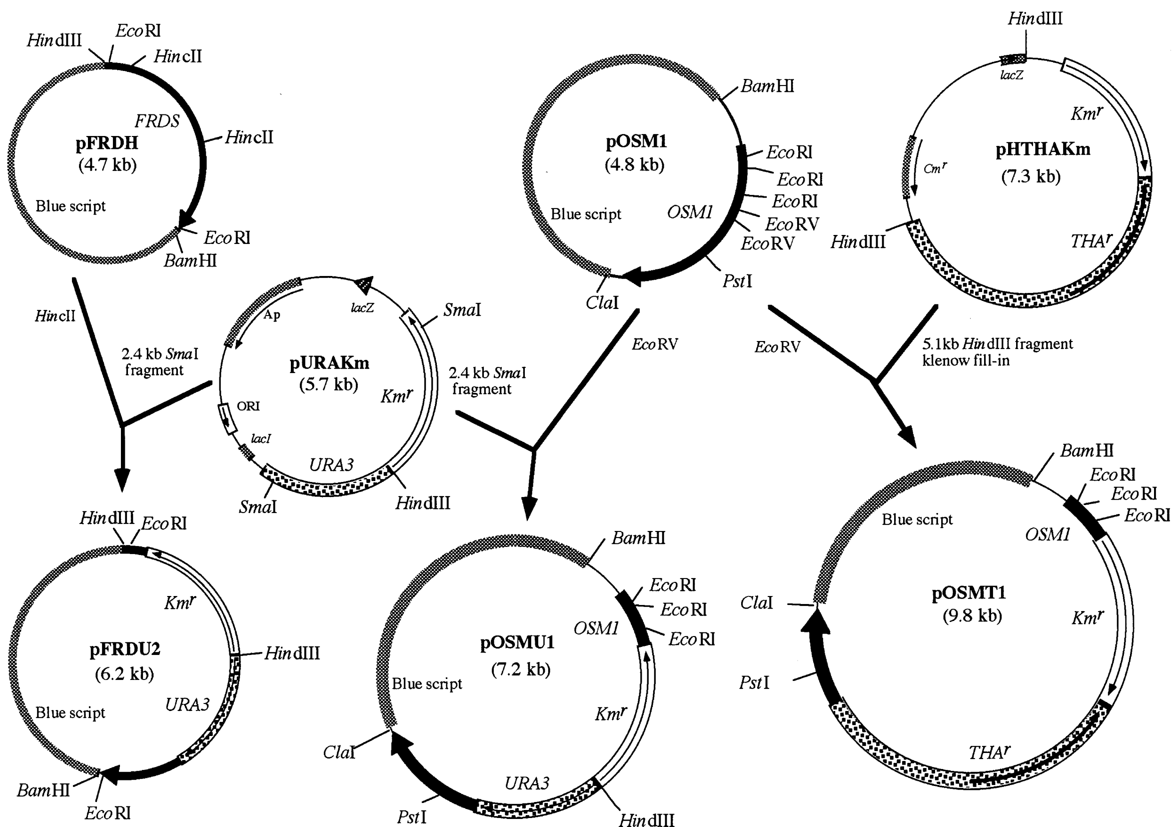

Fig. 1. Construction of plasmids for disruption of the *FRDS* and *OSM1* genes. The plasmid pURAKm was constructed by insertion of a 1.8-kb *Bam*HI fragment bearing the kanamycin resistant (Km^r) gene from the pNH-Kan/*ori*T plasmid [10], and a 1.3-kb *Hin*dIII fragment containing the *URA3* gene into the *Bam*HI and *Hin*dIII sites of pUC13, respectively. Plasmid pHTHAKm was constructed by the insertion of a 5.1-kb *Hin*dIII fragment containing the $ARO4^m$ gene (−1837 to +1427), which confers resistance to β-thienyl alanine (THA^r) [11], and the Km^r gene (inserted the blunt-ended 1.8-kb *Bam*HI fragment into the *Eco*RV site located 255 bp downstream from the stop codon of THA^r ORF) into pHSG398. Plasmid pFRDU2 was constructed for *FRDS* disruption. A 796-bp *Hin*cII fragment (+69 to +864) of the *FRDS* ORF was removed from pFRDH (the unique *Hin*cII site at the multirestriction site of plasmid pFRDS was deleted by *Hin*dIII-*Sal*I digestion) and replaced with a 2427-bp *Sma*I fragment containing the *URA3* and Km^r genes prepared from pURAKm. The plasmids pOSMU1 and pOSMT1 were constructed for OSM1 disruption. A 57-bp *Eco*RV fragment (+531 to +588) of the *OSM1* ORF was removed, and replaced, respectively, with a fragment containing the *URA3*-Km^r gene and a blunt-ended 5065-bp *Hin*dIII fragment containing the THA^r and Km^r genes from pHTHAKm.

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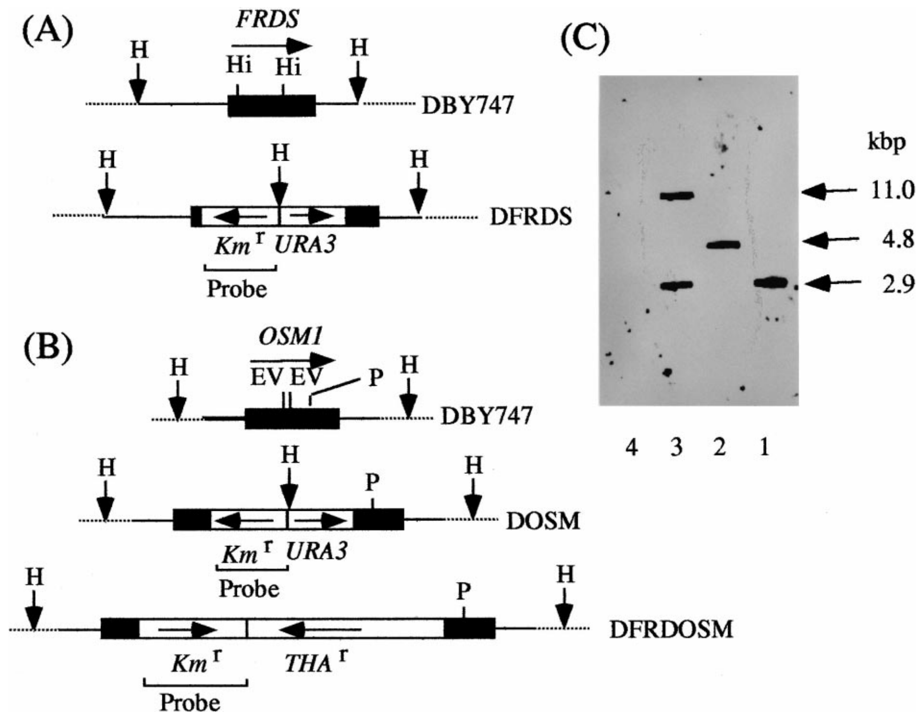

Fig. 2. Southern blot analysis of genomic DNAs from the wild-type strain and disruptants. The construction used to disrupt the *FRDS* (A) and *OSM1* (B) genes. The locations of the *Hin*dIII (H), *Hin*cII (Hi), *Eco*RV (EV) and *Pst*I (P) sites are marked. The *FRDS* and *OSM1* genes are depicted by solid bars. The arrows with the gene show the direction of transcription. Genomic DNAs were digested with *Hin*dIII, separated on 1% agarose gels, and then transferred onto a nylon membrane. The probe DNA was a 1.8-kb *Bam*HI fragment bearing the Km^r gene labeled with fluorescein. C: Lane 1, DFRDS; lane 2, DOSM; lane 3, DFRDOSM and lane 4, DBY747. A *Hin*dIII digest of lambda DNA was used as a size standard.

ated by agarose gel electrophoresis, and transferred onto a nylon membrane. When the 1.8-kb *Bam*HI fragment containing the Km^r gene from pNH-Kan/*ori*T plasmid was used as a probe, a single fragment corresponding to the expected size was detected for all transformants. Fig. 2C shows the presence of the expected 2.9-kb and 4.8-kb *Hin*dIII fragments in transformants DFRDS (lane 1) and DOSM (lane 2), respectively.

Next, the linear 6.9-kb fragment containing *OSM1::THA*^{r} was released from plasmid pOSMT1 (Fig. 1) by cleavage with *Bam*HI and *Cla*I, and used to transform DFRDS. Yeast transformants were selected on SD plates containing THA. A 11.0-kb *Hin*dIII fragment was detected as expected in DFRDOSM (Fig. 2C, lane 3), which was one of the 37 transformants examined by Southern hybridization.

Strain DFRDOSM, with disruption of both the *OSM1* and *FRDS* genes encoding FRDS2 and

Table 1
Fumarate reductase activitiy of the wild-type strain and fumarate reductase gene-disrupted strains

Strain	Genotype	Specific activity (μmol min^{-1} g $protein^{-1}$)
DBY747	*FRDS, OSM1*	6.7
DFRDS	*FRDS::URA3*	2.6
DOSM	*OSM1::URA3*	4.9
DFRDOSM	*FRDS::URA3, OSM1::THA*^{r}	Not detected

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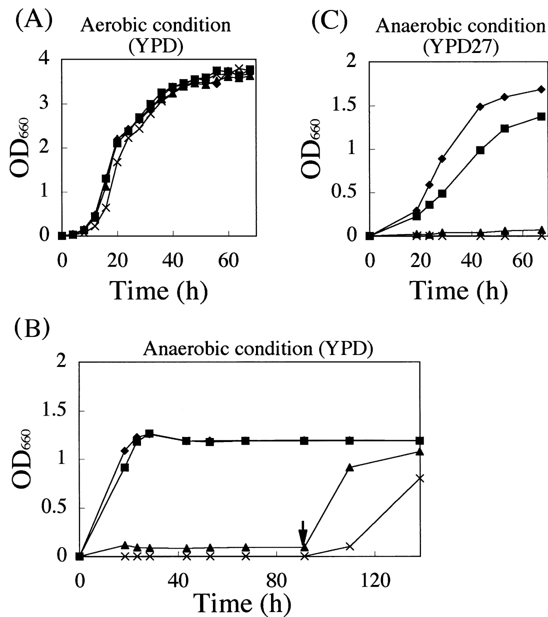

Fig. 3. Growth characteristics of the wild-type strain and the fumarate reductase gene disruptants on YPD medium under aerobic or anaerobic conditions. A: Aerobic condition maintained by shaking (40 rpm) in YPD. B: Anaerobic condition (static culture overlaid with liquid paraffin) in YPD. C: Anaerobic condition (static culture overlaid with liquid paraffin) in YPD27. Strains are (♦) DURA; (■) DFRDS; (▲) DOSM; and (×) DFRDOSM. After 90 h (time of the arrow) of anaerobic cultivation (B), liquid paraffin and 15 ml of culture were removed and the tubes were vigorously shaken for 5 min.

FRDS1, respectively, could not grow under anaerobic conditions as described later. Therefore, we determined the fumarate reductase activity in cells growing aerobically on 10% glucose, a condition that leads to repression of mitochondrial enzymes. The activities of the isoenzymes FRDS1 and FRDS2, in cells grown under aerobic conditions in a high concentration of glucose, were no different from those in cells grown under anaerobic conditions [17]. As summarized in Table 1, the fumarate reductase activity in the *FRDS*-disrupted strain, DFRDS, was 2.5-fold lower than that in the wild-type strain DBY747. This indicates that FRDS1, encoded by the *FRDS* gene, is the major fumarate reductase in *S. cerevisiae*. Fumarate reductase enzyme activity was absent in the double disruptant, DFRDOSM, indicating that *S. cerevisiae* does not have fumarate reductase-encoding genes other than *FRDS* and *OSM1*.

3.2. Growth properties of disruptants

As shown in Fig. 3, disruption of the *FRDS* and/or *OSM1* genes had no effect on aerobic growth. Singh et al. [7] reported that deletion of the *OSM1* gene results in inhibition of aerobic growth in hypertonic medium. However the Δ*osm1* disruptant, DOSM, could grow aerobically on 2 M glucose or 2% glucose in the presence of 2 M of ethylene glycol, glycerol or sorbitol (data not shown). The deletion mutant used by Singh et al. [7] lacked not only the *OSM1* gene but also the *CYC1* gene encoding iso-1-cytochrome *c*. Thus, the sensitivity to high osmotic conditions observed by them possibly results from the simultaneous inactivation of both the *OSM1* and *CYC1* genes, but not of the *OSM1* gene alone.

Disruption of both the *FRDS* and *OSM1* genes resulted in the failure of the yeasts to grow under anaerobic conditions in the presence of 2% or 27% glucose (Fig. 3). When the disruptants cultured under anaerobic conditions for 90 h were exposed to normoxia, they began to grow again, indicating that simultaneous inactivation of both the *OSM1* and *FRDS* genes during anaerobiosis is not lethal. The Δ*osm1*-deleted mutant grew slowly under anaerobic conditions. However, disruption of the *FRDS* gene had no effect under the same conditions. From these results, we conclude that cytosolic FRDS1 encoded by the *FRDS* gene and the promitochondrial FRDS2 encoded by the *OSM1* gene [6] are prerequisite more for growth under anaerobic conditions than for growth under hypertonic conditions.

Strain DFRDOSM was transformed using a yeast gene library (see Section 2). We obtained five independent transformants that were able to grow anaerobically. All transformants contained a plasmid bearing the *OSM1* gene (data not shown). This result strongly supports our conclusion that FRDS2 located in promitochondria is necessary for anaerobic growth.

We recently reported that the fumarate reductase isoenzyme FRDS2 encoded by the *OSM1* gene is located in the promitochodria of cells grown anaerobically [6]. The promitochondria lack an integrated electron transfer chain and a functional oxidative phosphorylation system [18]. Promitochondria are considered to be inactive for energy production. The energy required for anaerobic growth is pro-

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duced through anaerobic glycolysis. The present study strongly suggests that promitochondria containing FRDS2 are functional under anaerobic conditions. During anaerobiosis, oxygen is not available to oxidize reduced pyridine nucleotides on the respiratory chain coupled to oxidative phosphorylation. The resultant excess in reducing equivalents may be lowered through reactions catalyzed by FRDS1 and FRDS2. Ansell et al. [19] recently reported that the simultaneous disruption of yeast *GPD1* and *GPD2* genes which encode isoenzymes of NAD-dependent glycerol-3-phosphate dehydrogenase results in failure of anaerobic growth associated with intracellular accumulation of NADH.

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