

Cloning and Characterization of the *CSF1* Gene of *Saccharomyces cerevisiae*, Which Is Required for Nutrient Uptake at Low Temperature

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We have isolated cold-sensitive fermentation mutants (Csf mutants) of a commercial baker's yeast that have practically no fermentation capacity at 5°C and return to their normal capacity at 25 to 40°C. *CSF1* was cloned by functional complementation of the Csf phenotype. *CSF1* contain an open reading frame of 8,874 nucleotides, encoding a protein of 2,958 amino acids. The nucleotide sequence was identical to that of the YLR087C gene in the *Saccharomyces* genome database, but there was no information about the function of the predicted *CSF1* (YLR087C) protein. Gene disruption shows that *CSF1* is required for growth and fermentation only at low temperatures. Permeabilized cells of the disruptant showed nearly the same ethanol production rate as those of the parent strain, even at 10°C. The disruptant cells had the same glucose uptake rates as the parental cells at 30°C, but three- to fivefold-lower rates than the parental cells at 10°C. These findings suggest that *CSF1* associates with a new nutrient transport system which exists on the plasma membrane and is required only at low temperature.

Recently, we have established the refrigerated-dough process for bread making by using cold-sensitive fermentation (Csf) mutants of a baker's yeast. Some of them are now practically employed by bakeries in Japan. These Csf mutants produce much less CO₂ gas in dough than the parental strain at refrigeration temperatures but return to normal fermentation activity at room temperature or higher (7).

Cold-sensitive mutants of the yeast *Saccharomyces cerevisiae* have been reported, but they are mostly cold sensitive for growth, cell division cycling, or RNA splicing. One class of *cdc* mutants are well known as having a cold-sensitive cell division cycle. The *cdc50* mutant encoded a cold-sensitive mutation of the Cdc protein which was essential for growth at low temperature for an unknown mechanism (9; <http://genome-www.stanford.edu/Saccharomyces/>). The *LTE1* gene (16) was essential for growth at low temperature for an unknown mechanism. *NSR1* (6), a cold-shock-inducible gene, was reported as being related to growth at low temperature when cells were exposed to an abrupt temperature drop. *NSR1* protein was required for normal pre-rRNA processing. The *LTG3* gene (3) was essential for growth at low temperature only in a tryptophan auxotroph. The uptake of tryptophan seems to be a rate-limiting step in growth at low temperature. There are many other genes related to growth at low temperature. However, there was little information about the function of genes related to growth and fermentation only at low temperature.

In this study, we cloned *CSF1*, which complemented the *csf1* mutation in a baker's yeast strain. Our observations indicate

that *CSF1* is related to a new, unknown mechanism for nutrient uptake at low temperature.

MATERIALS AND METHODS

Strains and genomic libraries. RZT3 (7) is a *csf1* mutant strain derived from commercial baker's yeast strain KY5649 (Dia-Yeast YST; Kyowa Hakko Kogyo Co., Tokyo, Japan), and RZT3u is a *ura3* mutant of RZT3. YHK142 has the genotype *MAT α ura3 gal2 mal* (5). YHK1144 is a *CSF1* disruptant derived from YHK142 in this study. The library of genomic DNA used in this work contained DNA inserts of *Saccharomyces cerevisiae* X2180-1A and X2180-1B in the low-copy-number vector YCp50.

Media and genetic methods. YPD medium for cultivation contained 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% Bacto-peptone (Difco), and 2% glucose. SGLu was a synthetic medium containing 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco); when necessary, it was supplemented with appropriate nutrients. Buffered medium (BM) was SGLu medium with 100 mM potassium phosphate buffer (pH 5.0). Genetic manipulation was performed basically by the method of Matsuzaki et al. (10). Crude plasmid DNAs were prepared from yeast transformants by rapid glass bead disruption and phenol extraction (15). Contour-clamped homogenous electric field gel electrophoresis (CHEF) was performed by the method of Chu et al. (2) with CHEF DRII (Bio-Rad Laboratories, Richmond, Calif.). The protocols for labeling, hybridizing, and detecting DNA were described previously (4).

Plasmid construction. pHK162 is an originally cloned plasmid carrying *CSF1*. Plasmid pHK188, used for the production of URA⁺ cells carrying a disruption of the chromosomal *CSF1*, was constructed from pHK162 by inserting an 8-kb *Bam*HI-*Sau*3AI/*Bam*HI (nucleotides 1292 to 9589) fragment of *CSF1* from pHK162 into the *Bam*HI site of pUC19 and replacing a 0.6-kb *Mlu*I-*Spe*I (nucleotides 4379 to 5018) internal fragment of *CSF1* with the 1.2-kb *Hind*III-*Hind*III *URA3* fragment. Where necessary, DNA fragments were blunt-ended with the Klenow enzyme before the filled-in ligation.

Fermentation assay. The fermentative activity of yeast cells was measured as described previously (7).

DNA sequence analysis. DNA fragments were subcloned in the pUC19 vector, and a deletion set for DNA sequence determination was constructed using the Kilo-Sequence deletion kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's protocol. The sequencing reaction was done by the dideoxy chain termination method (12) with the T7 Sequencing kit (Pharmacia Biotech, Uppsala, Sweden). Both strands were sequenced by using the ALF DNA sequencer (Pharmacia Biotech) according to the protocol of the manufacturer. Computer analysis of the nucleotide and deduced amino acid sequences was performed with the DNASIS (Hitachi Software Engineering Co. Ltd., Yokohama, Japan). A

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homology search analysis of the deduced amino acid sequence was performed with the SWISS-PROT protein data bank.

Ethanol fermentation with permeabilized cells. Permeabilized cells were prepared as described by Takeshige and Ouchi (13). These cells were suspended in a buffer containing 5 mM MgCl₂, 2 mM ATP, 1 mM NAD⁺, 30 mM P_i, 1 mM arsenate, and 150 mM MES (morpholinioethanesulfonic acid, pH 6.9). These suspensions were preincubated at assay temperatures for 10 min. Ethanol fermentation was initiated by addition of glucose (final concentration, 2%). A portion (50 μl) of the reaction mixture was periodically withdrawn, and the ethanol content of the reaction mixture was measured with a gas chromatograph (Shimadzu GC-14A; Shimadzu Co., Kyoto, Japan). Ethanol fermentation in intact cells was assayed in the same conditions as for permeabilized cells.

Glucose uptake assay. The method for assay of glucose uptake was slightly modified from reference 14. Cells were grown in BM medium to an optical density at 600 nm of 3.0. Cells were collected by centrifugation, washed three times with 100 mM potassium phosphate buffer (pH 6.5), and suspended in the same buffer at a final concentration of 50 mg of cells (wet weight) per ml. Portions (50 μl) of the suspension and fivefold concentrates of ¹⁴C-labeled D-glucose (12.5 μl; purchased from Japan Radioisotope Association) were preincubated at the assay temperature and then mixed and incubated for 5 s, the period during which uptake was in the linear range. Uptake was terminated by addition of 10 ml of a quenching solution (100 mM potassium phosphate buffer [pH 6.5] containing 500 mM unlabeled glucose) at 0°C. Cells were then collected rapidly and washed on glass fiber filters (Whatman GF/F; purchased from Whatman Japan Ltd., Tokyo, Japan) with 20 ml of the quenching solution. Filters were placed in 3.5 ml of scintillant, and radioactivity was measured with a Beckman LS335 liquid scintillation counter. The control blank in each experiment consisted of labeled glucose added to the quenching solution before the yeast cells were added. The glucose concentration for the experiment ranged from 0.1 to 100 mM (specific radioactivity, 6 to 740 kBq/μmol).

Leucine uptake assay. Leucine uptake was measured by the method of Ramos et al. (11). ¹⁴C-labeled L-leucine was purchased from the Japan Radioisotope Association. The conditions for the leucine uptake assay were the same as those for the glucose uptake assay except for the incubation time (1 min) and the quenching solution (distilled water).

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of *CSF1*. The genomic library was screened for a gene which complemented the *csf1* mutation using RZT3u as the host strain by a color plate assay (7). Out of about 40,000 colonies, one positive clone was obtained that carried a plasmid named pHK162. pHK162 contained a 12-kb inserted fragment. Reintroduction of the plasmid into RZT3u restored wild-type fermentation at 10°C. The complete nucleotide sequence of the 12-kb inserted fragment was determined on both strands by using overlapping deletion mutants. The fragment contained a sole open reading frame (ORF) of 8,874 bp that encoded a polypeptide of 2,958 amino acids with a calculated molecular mass of 338 kDa. The complementation analysis with various deletion plasmids indicated that this ORF encoded *CSF1* (data not shown). The nucleotide sequence was identical to that of the YLR087C gene in the *Saccharomyces* genome database (<http://genomewww.stanford.edu/Saccharomyces/>). YLR087C is located on chromosome XII. This information was coincident with chromosome mapping data for the *csf1* mutation which we had obtained by a specific chromosome loss technique (4, 5) (data not shown). The predicted CSF1 (YLR087C) protein contained four transmembrane motifs, but there was no information about the function of the protein. When the amino acid sequence of CSF1 was compared with available protein sequences in the GenBank, SwissProt, PIR, PRF, and TIGR databases using TBLASTN (1), only *Candida albicans* Con4-3103 (positions 41995 to 33164) in the TIGR database was found to be similar to CSF1 (29% identity, 48% similarity). However, the TIGR database is constructed with unfinished microbial genome sequences and still contains errors. There was no information about the function of this gene in *C. albicans*.

We failed to detect *CSF1* mRNA in wild-type cells cultured at both 10 and 30°C by Northern blotting hybridization analysis (data not shown), presumably because the transcription level of *CSF1* was below detection.

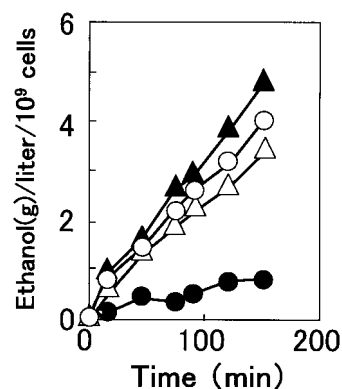


FIG. 1. Ethanol production by permeabilized (open symbols) and intact (solid symbols) cells of the parent strain YHK142 (triangles) and the disruptant strain YHK1144 (circles) at 10°C. Cells were suspended in a buffer containing 5 mM MgCl₂, 2 mM ATP, 1 mM NAD⁺, 30 mM P_i, 1 mM arsenate, and 150 mM MES (pH 6.9). Ethanol fermentation was initiated by addition of glucose (final concentration, 2%).

Phenotypes of a *CSF1* disruptant strain. Plasmid pHK188 was digested with *Bam*HI, and the linear fragment containing *CSF1::URA3* obtained was integrated into the *CSF1* allele in strain YHK142. Several yeast transformants prototrophic for uracil were selected on SGLu plates. One of these strains, YHK1144, was used for the following experiments. The *CSF1* disruption in strain YHK1144 was confirmed by Southern blot analysis of genomic DNA from strains YHK1144 and YHK142 with a *Bam*HI-*Sph*I 6.4-kb DNA fragment (nucleotides 1292 to 7670) of pHK162 as a probe (data not shown). These findings indicate that *CSF1* is not essential for cell growth under normal conditions.

The shape and size of cells, mating ability (*MAT*α), and growth rate at 30°C were indistinguishable between YHK1144 and YHK142. When cultured at 10°C, however, YHK1144 cells did not apparently grow even after 170 h, while the parental cells grew and reached maximum growth in 120 h (data not shown). These results indicate that *CSF1* is required for cell growth at low temperature.

Growth of the *CSF1* disruptant YHK1144 and the *csf1* mutant RZT3 at 30°C was more sensitive to hygromycin B than that of either parent strain, but no difference was observed in the sensitivity to vanadate (data not shown).

Ethanol production in permeabilized cells. Permeabilized cells are those which have lost their plasma membrane semi-permeability but whose enzymes remain virtually intact within the cells or cellular boundary. Therefore, by comparing the fermentation activities of intact and permeabilized cells at low temperature, it may be possible to determine the sites in cells which are cold sensitive for fermentation. As shown in Fig. 1, permeabilized cells of YHK142 showed nearly the same ethanol production rate as those of YHK1144 even at 10°C. This finding was quite different from that in the control experiment, in which intact cells of YHK1144 showed a fivefold-lower rate than those of YHK142. These results suggested that the primary site of cold-sensitive fermentation was not inside the cells but on the plasma membrane.

Kinetics of glucose and leucine uptake. In *S. cerevisiae*, glucose uptake is mediated by low- and high-affinity transport systems (*K_m*s of approximately 20 and 1 mM, respectively). Therefore, we examined the kinetics of glucose uptake by YHK142 and YHK1144 in the range from 1 to 100 mM D-glucose at 10 and 30°C. YHK1144 showed the same uptake rates as YHK142 at 30°C but three- to fivefold-lower rates than

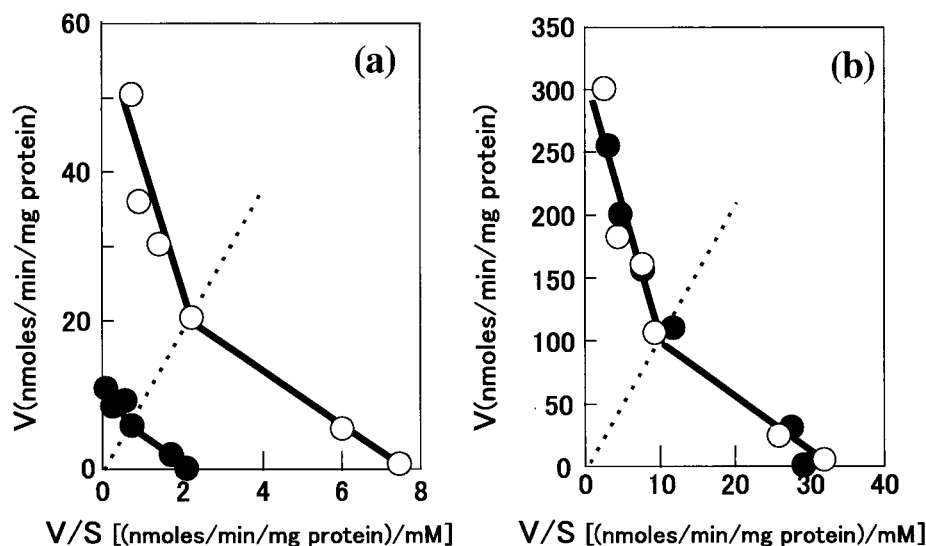


FIG. 2. Eadie-Hofstee plots of glucose transport in wild-type strain YHK142 (○) and *CSFI* disruptant strain YHK1144 (●) at 10°C (a) and 30°C (b). The glucose concentration ranged from 0.1 to 100 mM (specific radioactivity, 6 to 740 kBq/ μ mol). The assay mixture was incubated for 5 s, the period during which uptake was in the linear range. Dashed lines represent a substrate concentration of 10 mM. Data were obtained from duplicate experiments.

YHK142 at 10°C at any glucose concentration tested (Fig. 2). These results indicated that both low- and high-affinity transport systems for glucose were cold sensitive in YHK1144.

We estimated activation energies for the transport of glucose based on the kinetic data from uptake assays with 20 mM glucose at temperatures from 5 to 40°C. As shown in Fig. 3, there was a break point on each slope of the Arrhenius plots at about 25°C. The activation energies calculated from the slopes were 5 and 8 kcal/mol for YHK142 and YHK1144, respectively, below 25°C, but there was no difference above 25°C.

Activation energies were also different for L-leucine transport above 25°C between YHK142 and YHK1144, 12 and 17 kcal/mol, respectively. Break points were again found at about 25°C, and above 25°C there was no difference in L-leucine uptake rates (Fig. 3).

In addition, our preliminary experiment showed that RZT3, a *csf1* mutant, exhibited cold-sensitive maltose fermentation (data not shown). These findings suggest that *CSFI* is required for the transport not only of glucose but also of other nutrients at low temperature in *S. cerevisiae*.

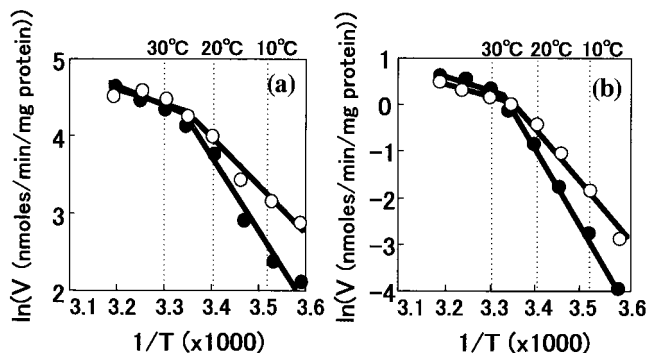


FIG. 3. Arrhenius plots of glucose (a) and L-leucine (b) transport at various temperatures in wild-type strain YHK142 (○) and *CSFI* deletion mutant YHK1144 (●). Glucose transport was measured for 5 s with 20 mM glucose. L-Leucine transport was measured for 1 min with 1 mM L-leucine. The experiment was repeated twice.

It is clear that the function of *CSFI* is different from that of any previous reported genes, but the mechanism by which *CSFI* associates with the nutrient transport systems at low temperature is still unknown. The lipid composition of the plasma membrane and H^+ -ATPase activity are well known as important factors affecting membrane potential and most transport systems. Recently, many hygromycin B-sensitive mutants were reported, and some mutations affected membrane potential (8). The *CSFI* disruptant strain was more sensitive to hygromycin B than the parent strain. Therefore, it was deduced that the glucose uptake activity of *CSFI* disruptants decreased as plasma membrane H^+ -ATPase activity decreased. However, our preliminary data showed no difference in lipid composition and plasma membrane ATPase activity at 10°C between YHK142 and YHK1144 (data not shown).

To understand the actual functions of *CSFI* or the Csf1 protein, further studies are required: (i) cloning and sequence analyses of the mutant *csf1* gene of RZT3, (ii) analyses of transcription level and transcriptional control of *CSFI* by using a reporter gene connected to the *CSFI* promoter or a multicopy plasmid carrying *CSFI*, and (iii) determination of the location of Csf1 protein with a specific antibody.

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