Characterization of a *Saccharomyces cerevisiae* Mutant with Pseudohyphae and Cloning of a Gene Complementing the Mutation

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Screening for morphological mutants of a haploid strain of *Saccharomyces cerevisiae* was done on the basis of their cell-shape on a solid medium containing isoamyl alcohol, which causes cell elongation, to obtain information on the morphogenesis. Mutant J19, which had pseudohyphae in liquid medium even in the absence of isoamyl alcohol, had many elongated cells. Few reports exist of haploid cells growing as pseudohyphae in liquid culture. Cell-wall analysis showed that J19 had ordinary amounts of alkali-insoluble glucan and chitin, but that isoamyl alcohol in the medium caused structural changes in the cell wall. Addition of a DNA fragment that included the wild-type *SCL1* gene to J19 complemented its morphological phenotype. Sequencing of J19 *SCL1* showed that the glycine at position 226 in the Scl1 protein had been replaced by asparatic acid, suggesting that this mutation in the protein, a subunit of proteasomes, may be involved in the morphological change.

**Key words:** pseudohyphal growth; cell extension; cell wall; *SCL1*; isoamyl alcohol

The budding yeast *Saccharomyces cerevisiae* can differentiate into a form similar to that of *Candida albicans*. Usually *S. cerevisiae* grows in a yeast-like form, but under conditions of nitrogen limitation, diploid cells grow as long, branched chains of cells, which are called pseudohyphae. In nutritionally rich agar growth media, haploid cells also have a growth form in which they penetrate the agar. To date, many *S. cerevisiae* genes involved in pseudohyphal and invasive growth have been identified, as reviewed elsewhere. Several fusel alcohols cause these morphological changes to pseudohyphal or aberrant elongated shape in both haploid and diploid strains of *S. cerevisiae*. The aberrant shape resembles that of germ tubes of *C. albicans*. Dickinson discovered this effect and called the elongated shape a hyphal-like extension. Yeast strains vary in their susceptibility to such alcohols.

We earlier examined several strains for hyphal-like extension on rich agar medium and found that isoamyl alcohol causes cell elongation in the *pkcl* deletion mutant at a high frequency. However, the relationship between the elongation and the Pkc1 protein has been difficult to establish because this protein has many functions. Recently, Ashe et al. found that 1-butanol and isoamyl alcohol inhibit translation of the protein at the step of initiation. Some environmental stimuli cause morphological changes via a signal transduction pathway. The change to pseudohyphal growth is controlled by two signal transduction pathways, one a mitogen-activated protein kinase cascade, the other a cAMP-dependent pathway. Lorenz et al. reported that the cascade controls alcohol-induced morphological changes. Little information is available on the later part of these pathways, except for the *MUC1*/*FLO11* gene. This gene encodes a cell-surface glycoprotein that is a target for both of these pathways; the glycoprotein is needed for pseudohyphal and invasive growth. The purpose of this study was to identify other factors than Flo11 protein and to investigate their involvement in the transformation to either pseudohyphal forms or else to the hyphal-like extension. A screen for morphological mutants identified a mutant, J19 that has pseudohyphal growth in both liquid and solid media. The phenotype of this mutant was examined. The morphological phenotype was complemented by the *SCL1* gene (suppressor of cycloheximide resistant, temperature-sensitive lethal mutation), which is essential for growth. The Scl1 protein Sc1p is a subunit of proteasomes, which degrade proteins in a ubiquitin-dependent way. Here we attempted to elucidate the relationship between Sc1p and the change to the pseudohyphal shape.
Materials and Methods

Strains and media. S. cerevisiae strains W303-1A (MATalpha ura3-1 trpl-1 ade2-1 leu2-3,113 his3-11,15 can1) and SH985 (MATalpha trpl his3 lys2) were used. Cells were cultured at 30°C with the following culture media: YPD (1% yeast extract, 2% peptone, and 2% dextrose), YPDS (YPD with 1.0 M sorbitol), 1/5 YPD (0.2% yeast extract, 0.4% peptone, and 0.4% dextrose), 1/5 YPDS (1/5 YPD with 1.0 M sorbitol), SD (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose, and 5 g/l ammonium sulfate), and synthetic low-ammonia dextrose (SLAD; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose, and 6.6 mg/l ammonium sulfate), and synthetic low-ammonia dextrose (SLAD; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose, and 6.6 mg/l ammonium sulfate) for nitrogen starvation. Solid media contained 2% agar. Escherichia coli strain JM109 was used for construction and propagation of plasmids.

Mutagenesis and screening. Cells of the W303-1A strain were treated with ethyl methanesulfonate at concentrations that gave 5% to 20% survival, and then cells were incubated on YPDS plates at 30°C for 2 to 3 days. Colonies were transferred with a toothpick to 1/5 YPD and 1/5 YPDS plates and growth rates were compared on the two media. After another 2 to 3 days, mutants that grew faster on 1/5 YPDS than on 1/5 YPD were selected. The selected strains were cultured on YPDS plates at 30°C for 2 to 3 days and transferred to YPDS plates containing 0.5% (v/v) isoamyl alcohol, because we previously had found this concentration to cause hyphal-like extensions in the pkc1 deletion mutant. Cells were incubated at 30°C for 24 to 48 h, picked up with a toothpick, suspended in 1.0 M sorbitol on glass slides, and examined for cell extension. An elongated cell was defined as one 3 times as long as an ordinary cell and examined for cell extension. An elongated cell was obtained from the cell-wall fraction as described by Dijkgraaf et al. and digested with Zymolyase 100-T (Seikagaku Corp., Japan). After dialysis, β-1,6-glucan was collected, and the hexose content was measured. Total alkali-insoluble glucan was measured as the hexose content before dialysis, and the β-1,3-glucan level was calculated by subtraction of the β-1,6-glucan content from total glucan.

Sensitivity test. To evaluate sensitivity because of cell-surface defect to SDS and hygromycin B, cells were cultured in 3 ml of liquid YPDS at 30°C overnight. The cell concentration was adjusted to an OD600 of 0.50 ± 0.01, and a 10-fold serial dilution was made. Three microliters of the cell suspension was put on the surface of YPDS plate containing SDS (0.0005%–0.03%) or hygromycin B (10–100 μg/ml), and the plates were incubated at 30°C for 24 to 72 h. To evaluate ethanol sensitivity, cells were cultivated in liquid YPDS containing ethanol (2.5%–15%) at 30°C, and the OD600 of the culture was measured. Temperature sensitivity of the mutant J19 was tested in cells grown on YPDS plate at 18, 25, 30, and 37°C.

Plasmids and genetic analysis. Plasmids pRS316 (URA3 and CEN6) and YCp50 (URA3 and CEN4) were used as vectors. Plasmid pRS316-PKC1, constructed by Kohno et al., was used for the complementation test. To find if the mutation in J19 was recessive, strain J19 (MATalpha ura3-1 trpl-1 ade2-1 leu2-3,113 his3-11,15 can1) was crossed with SH985. The diploid strain grown on SD plate containing tryptophan and histidine was obtained. The strain did not have pseudohyphae and isoamyl alcohol did not cause the cell extension in the strain.

Yeast transformation was done with lithium acetate. A mutant was transformed with 1 μg of a genomic library (ATCC 37415) for screening for complementary genes. Five hundred strains that grew faster than J19 were selected from the transformants. Next, thirty-eight strains that grew on 1/5 YPD plate almost as fast as on 1/5 YPDS plate was selected. Similar experiments were done on SD and SD containing 1 M sorbitol plates. We found that a strain grew in a yeast-like form and did not respond to isoamyl alcohol with cell extension. The plasmid was isolated from the transformant. Partial sequencing of the insert DNA and identification of restriction enzyme cleavage sites showed that the plasmid contained a 6.2-kb Sau3AI fragment (bearing the complete SCL1 and ERG4 sequences and the incomplete PDR1 gene, all originating from chromosome VII) inserted into the BamHI site of YCp50. Next,
subcloning of each gene was done. The plasmids pRS316-SCL1, pRS316-ERG4, and pRS316-PDR1 were constructed by the insertion Clal-Sacl, EcoRI, and SacI-SalI fragments into the Clal-Sacl, EcoRI, and SacI-SalI sites of the plasmid pRS316, respectively (see Fig. 5).

Sequencing was done with an ABI 377 DNA sequencer at Takara Bio Inc. Otsu Japan. SCL1 gene sequences of the mutant J19 and the parental strains were obtained by sequencing of the PCR products with Takara Ex Taq. Sequences were compared with those in the Saccharomyces Genome Database.

Results

Screening for morphological mutants

Cell extension was uncommon in the W303-1A cells. In the pkc1 deletion mutant, which has defective cell walls and is osmotically fragile, the percentage of cells with extensions was higher than in parental-strain cells. Forty strains were selected in which the addition of sorbitol to the medium allowed normal or near-normal cell growth. Screening for mutants with a large proportion of hyphal-like extensions after isoamyl alcohol treatment was done. The shapes of cells grown on YPDS plate with 0.5% isoamyl alcohol were examined. In eight of the 40 mutants, a larger percentage of cells had extensions than with the parental strain (Table 1). The proportions were 20% or more for mutants J9, J10, and J19, with 37.4% of J19 cells having an extension.

Table 1. Effects of Isoamyl Alcohol on Cell Morphology in Mutants on YPDS Plate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isoamyl alcohol (0.5% v/v)</th>
<th>Cell extension (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>W303-1A</td>
<td>–</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.8±1.9</td>
</tr>
<tr>
<td>J6</td>
<td>–</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18.7±3.8</td>
</tr>
<tr>
<td>J9</td>
<td>–</td>
<td>9.9±2.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>29.5±10.7</td>
</tr>
<tr>
<td>J10</td>
<td>–</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20.5±1.7</td>
</tr>
<tr>
<td>J17</td>
<td>–</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.2±0.5</td>
</tr>
<tr>
<td>J19</td>
<td>–</td>
<td>4.5±0.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20.4±2.8</td>
</tr>
<tr>
<td>J28</td>
<td>–</td>
<td>0.3±0.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16.8±0.4</td>
</tr>
<tr>
<td>J37</td>
<td>–</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.6±1.0</td>
</tr>
<tr>
<td>J39</td>
<td>–</td>
<td>11.6±1.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16.4±4.3</td>
</tr>
</tbody>
</table>

Cells were cultivated on YPDS plate with or without isoamyl alcohol at 30°C. Cells were picked up with a toothpick and suspended in 1.0M sorbitol on a glass slide. Cell extensions were measured by counting of at least 300 cells. All values are means of three independent determinations ± SD.

The J19 morphological phenotype was not complemented by PKC1.

Recently Lorenz et al. reported that S. cerevisiae strain Σ 1278 grown on solid SLAD medium containing 1.0% butanol has cylindrical, elongated cells or round yeast-like cells.10 Growing the J19 and parental strains on solid SLAD medium with isoamyl alcohol resulted in cell extensions of the cells, in 69.2% and 32.1%, respectively (Table 2). We chose J19 for further characterization.

Table 2. Effects of Isoamyl Alcohol on Cell Morphology in J19 and the Parental Strain on SLAD Plate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isoamyl alcohol (0.5% v/v)</th>
<th>Cell extension (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (h)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>W303-1A</td>
<td>–</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>56.5±6.1</td>
</tr>
<tr>
<td>J19</td>
<td>–</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18.4±3.3</td>
</tr>
</tbody>
</table>

Cells were cultivated on SLAD plate with or without isoamyl alcohol at 30°C. All values are means of three independent determinations ± SD.

Results

Screening for morphological mutants

Cell extension was uncommon in the W303-1A cells. In the pkc1 deletion mutant, which has defective cell walls and is osmotically fragile, the percentage of cells with extensions was higher than in parental-strain cells. Forty strains were selected in which the addition of sorbitol to the medium allowed normal or near-normal cell growth. Screening for mutants with a large proportion of hyphal-like extensions after isoamyl alcohol treatment was done. The shapes of cells grown on YPDS plate with 0.5% isoamyl alcohol were examined. In eight of the 40 mutants, a larger percentage of cells had extensions than with the parental strain (Table 1). The proportions were 20% or more for mutants J9, J10, and J19, with 37.4% of J19 cells having an extension.

![Figure 2](image-url)
Cell-wall structure
J19 did not grow in media containing either 0.003% SDS or 30 μg/ml hygromycin B, but the parental strain grew in these media. The concentrations of β-1,6-glucan and β-1,3-glucans in the cell walls when isoamyl alcohol was not added to the medium were the same in the parental and J19 strains (Table 3). Next, we examined the effects of isoamyl alcohol on cell-wall composition. The addition of isoamyl alcohol to the medium decreased the levels of β-1,6- and β-1,3-glucans in both parental and J19 strains. Staining with calcofluor white showed no difference in the amounts of chitin in pseudohyphal

J19 cells and yeast-like parental-strain cells. The pseudohyphal cells stained strongly only at their septa (Fig. 3).

Other J19 phenotypes
J19 grew slowly on media without sorbitol, but growth was slightly less slow when 1.0 M sorbitol was present. J19 grew more slowly at 37°C than at 30°C, but the parental strain grew normally at 37°C. J19 grew more slowly with 2.5% ethanol, but at this con-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isoamyl alcohol 0.4% (by vol.)</th>
<th>Most common cell shape</th>
<th>Alkali-insoluble glucan concentration: μg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total glucan</td>
</tr>
<tr>
<td>W303-1A</td>
<td>–</td>
<td>Yeast-like</td>
<td>310.3 ± 12.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Pseudohyphal and hyphal-like</td>
<td>174.6 ± 11.2</td>
</tr>
<tr>
<td>J19</td>
<td>–</td>
<td>Pseudohyphal</td>
<td>298.8 ± 29.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Hyphal-like</td>
<td>179.7 ± 7.9</td>
</tr>
</tbody>
</table>

All values are means of three independent determinations ± SD. Glucan concentrations are expressed as microgram of glucan per milligram (dry weight) of the cell wall. –, not added; +, added to the final concentration shown.
centration growth of the parental strain was not affected. When J19 cells were grown on YPDS plate with isooamyl alcohol and stained with DAPI, the elongated cells were multinucleate (Fig. 4).

*Genes complementing J19*

Analysis of the strain obtained by crossing with SH985 showed that the mutation in J19 was recessive. By screening of 2,300 transformants, we obtained one strain, without the hyphal-like extensions and pseudohyphal growth of untransformed J19. When J19 was transformed with three plasmids (pRS316-SCL1, pRS316-ERG4, and pRS316-PDR1; Fig. 5), only in the cells transformed with pRS316-SCL1 was there no hyphal-like extensions on YPDS containing isooamyl alcohol, nor was pseudohyphal growth observed. *SCL1* seemed to suppress the J19 morphological phenotype.

One difference in the *SCL1* sequences of the J19 and parental strain was found in the open reading frame, in which the base at position 677 was adenine in J19 but guanine in both the parental strain and the *Saccharomyces* database. The mutation would change the wild-type Gly-226 codon (GGT) to an Asp codon (GAT).

**Discussion**

The observation that a high proportion of morphological variants (8/40) with a high isoamyl
alcohol-induced ratio of hyphal-like forms are obtained from osmotically fragile mutants may reflect the strong effect of cell wall structure on cell morphology.

In mutant J19 the ratio of cell extensions induced by isoamyl alcohol were high (37.4%), whereas nitrogen starvation increased induction of cell extension in both the parental strain (32.1%) and J19 (69.2%). This result suggests that nitrogen starvation and the J19 mutation may interact synergistically in the isoamyl alcohol-induced production of this phenotype. The J19 mutation may be involved in the induction at a point different from that of the nitrogen-starvation pathway. There are few reports on the hyphal-like extension, and the extension mechanism is unclear. The result of DAPI staining showed that the J19 cells elongated by addition of isoamyl alcohol was multinucleate. Elongated cells of the J19 cells elongated by addition of isoamyl alcohol is unclear. The result of DAPI staining showed that hyphal-like extension, and the extension mechanism is unclear. The result of DAPI staining showed that the J19 cells elongated by addition of isoamyl alcohol was multinucleate. Elongated cells of the J19 cells elongated by addition of isoamyl alcohol were not observed in the elongated cells. Addition of isoamyl alcohol may inhibit the formation of the septum-like structure. Analyses with J19 will contribute to our understanding of this mechanism.

The pseudohyphal growth of J19 in both YPDS and SLAD liquid media without isoamyl alcohol indicates that J19 is useful for investigating pseudohyphal growth and the morphological transition. J19 cells have slightly elongated morphology in comparison with the parental strain. Generally haploid cells bud in an axial pattern. However as a result of observation of cell morphology, it seems that many pseudohyphal cells in J19 bud in a unipolar fashion. This result is similar to features reported in the pseudohyphal growth of diploids. Also adhesion between the different pseudohyphae was not observed.

Experiments with SDS and hygromycin B sensitivities suggested a defect in the cell wall, however, no abnormalities were noted in glucan and chitin for J19. It is possible that this mutation may play important roles in the morphological change at a specific stage in cell-wall synthesis or degradation. The result of calcofluor white staining showed that the chitin mainly existed at their septa in J19. The mutation in J19 may lead to loss of the function for cell separation. The answers to these questions await future work.

Chitin levels in mutants with defective synthesis of β-1,3- or β-1,6-glucan are higher than in parental strains, and diploid cells with pseudohyphal growth under stressful conditions accumulate chitin on the cell surface. Staining with calcofluor white cells showed no difference in the amounts of chitin in J19 and parental-strain cells. These results may possibly be due to the differences between our study and the former in terms of culture conditions or in ploidy.

The addition of isoamyl alcohol decreases β-1,3-glucan and β-1,6-glucan levels in the cell wall of both J19 and parental strains. The β-1,6-glucan level of J19 cultured with the alcohol was higher (61.7 μg/mg) than that of the parental strain (40.0 μg/mg), even though the proportion of cells with hyphal-like extensions in J19 was higher than in the parental strain (not shown). Consequently it is difficult to relate the observed changes in β-1,6-glucan levels to the transformation in morphology. Isoamyl alcohol-induced cell extensions have been observed in the pkcl deletion mutant, which has a defect in β-1,3-glucan synthesis. Considering these results, decreasing β-1,3-glucan levels may be important for inducing cell extension.

The SCL1 gene complements the J19 morphological phenotype. The proteasome appears to be present in all eukaryote species and catalyzes ubiquitin-dependent proteolysis. The proteasome, required for the proteolysis of short-lived and misfolded proteins, plays a role in the control of cell proliferation and the cell cycle. The proteasome (26S) consists of a 19S regulatory portion and a 20S proteolytically active portion, the structure of which is known from X-ray crystallographic studies. The 20S proteasome, consisting of two outer α-rings and two inner β-rings, has at least three different peptidase activities (a chymotrypsin-like, a trypsin-like, and a peptidyl-glutamyl peptide-hydrolyzing activity). The Scl1 protein is a subunit of the α-ring, which consists of seven different subunits. It is possible that proteasome proteolysis is involved in the morphological change between the yeast-like and pseudohyphal forms. In the future, it is necessary to analyze accumulation of ubiquitinated proteins in J19 cells and the effects of the mutation in Scl1p on the three peptidase activities of the proteasome.

The Gly residue at position 226 in wild-type Scl1p was replaced by an Asp residue in the J19 mutant. The NCBI Blast program was used to search for proteins similar to Scl1p. A number of proteins have substantial sequence similarity, including those of C. albicans Orf6.4140p (64% similarity), Schizosaccharomyces pombe Spbc646.16p (56%), Mus musculus Psmα6p (55%), and Rattus norvegicus Psmα6p (55%). The Gly 226 residue present in S. cerevisiae Scl1p is conserved in all of these proteins, suggesting that Gly 226 is important for Scl1p function. In addition, this mutation apparently only partially impairs the function of Scl1p because the protein is known to be essential for growth.

Heretofore, some proteasome mutants are obtained. As far as we know, morphological abnormality has not been reported in mutants of subunits, which consist of α-ring. However in mutants of the β-ring and 19S regulatory particle, the abnormality is known. Those reports also suggest that the proteasome is related to the cell morphology.
Acknowledgments

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References

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