

# A Baker's Yeast Mutant (*fil1*) With a Specific, Partially Inactivating Mutation in Adenylate Cyclase Maintains a High Stress Resistance During Active Fermentation and Growth

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## Abstract

The initiation of fermentation in the yeast *Saccharomyces cerevisiae* is associated with a rapid drop in stress resistance. This is disadvantageous for several biotechnological applications, e.g. the preparation of freeze doughs. We have isolated mutants in a laboratory strain which are deficient in fermentation-induced loss of stress resistance ('fil' mutants) using a heat shock selection protocol. We show that the *fil1* mutant contains a mutation in the *CYR1* gene which encodes adenylate cyclase. It causes a change at position 1682 of glutamate into lysine and results in a tenfold drop in adenylate cyclase activity. The *fil1* mutant displays a reduction in the glucose-induced cAMP increase, trehalase activation and loss of heat resistance. Interestingly, the *fil1* mutant shows the same growth and fermentation rate as the wild type strain, as opposed to other mutants with reduced activity of the cAMP pathway. Introduction of the *fil1* mutation in the vigorous Y55 strain and cultivation of the mutant under pilot scale conditions resulted in a yeast that displayed a higher freeze and drought resistance during active fermentation compared to the wild type Y55 strain. These results show that high stress resistance and high fermentation activity are compatible biological properties. Isolation of *fil*-type mutations appears a promising avenue for development of industrial yeast strains with improved stress resistance during active fermentation.

## Introduction

In the yeast *Saccharomyces cerevisiae* an opposite relationship exists between stress resistance and fermentation activity. Yeast cells growing on nonfermentable carbon sources and stationary-phase cells display a high stress resistance. Cells growing on glucose or other rapidly-fermented sugars, on the other hand, have a much lower stress resistance (Plesset *et al.*, 1987; Schenberg-Frascino and Moustacchi, 1972). The initiation of fermentation after addition of glucose is invariably associated with a rapid drop in stress resistance (Van Dijck *et al.*, 1995).

The low stress resistance during active fermentation of yeast is disadvantageous for its use in industrial applications (Attfield, 1997). A striking example is the preparation of freeze doughs. When the flour is mixed with the yeast for the preparation of the dough, the yeast initiates fermentation and at the same time the freeze resistance of the yeast rapidly drops (Nagodawithana and Trivedi, 1990; Rose and Vijayalakshmi, 1993). To minimize the problem, freeze doughs are prepared by rapid mixing of the dough at low temperature. However, a minimal prefermentation of the dough appears to be necessary for a good quality of the bread (Hsu *et al.*, 1979a; Hsu *et al.*, 1979b; Richard-Molard *et al.*, 1979). As a result of the drop in dough rising capacity during preparation of freeze doughs more yeast has to be added which has a negative influence on the taste of the bread.

In *S. cerevisiae* the cAMP-PKA pathway exerts a strong influence on cellular stress resistance. Yeast mutants with elevated activity of the pathway display a lower stress resistance while mutants with reduced activity of the pathway display enhanced stress resistance (Cameron *et al.*, 1988; Park *et al.*, 1997; Sass *et al.*, 1986; Shin *et al.*, 1987a; Toda *et al.*, 1987a; Toda *et al.*, 1987b). However, the cAMP-PKA pathway also influences the growth rate of the cells. Inactivation of the pathway causes growth arrest in the G1 phase of the cell cycle followed by permanent entry into the stationary phase G0 (Ishikawa *et al.*, 1986; Martegani *et al.*, 1986; Shin *et al.*, 1987b). Reduction of adenylate cyclase or PKA activity leads to a reduction in the growth rate of the cells, a much longer lag phase and a reduced fermentation rate (Ma *et al.*, 1997). Hence, it did not appear to be a promising approach to enhance stress resistance in industrial yeast strains. In this paper, however, we describe a specific mutation in adenylate cyclase that reduces its activity to such an extent that stress resistance is significantly enhanced without negatively affecting the growth and fermentation rate.

Addition of glucose to yeast cells growing on a nonfermentable carbon source or stationary phase cells triggers a spike in the cAMP level which sets off a PKA-

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mediated protein phosphorylation cascade with a rapid drop in stress resistance as one of the physiological consequences (Reviews: Thevelein, 1991; Thevelein and de Winde, 1999). Recent work has shown that glucose activation of the cAMP pathway involves a G-protein coupled receptor system, consisting of a serpentine-receptor like protein, Gpr1, and a heterotrimeric G $\alpha$  protein, Gpa2 (Colombo *et al.*, 1998; Kraakman *et al.*, 1999). However, glucose phosphorylation is also required in some way for activation (Beullens *et al.*, 1988). Mobilisation of trehalose (Thevelein and Beullens, 1985; van der Plaats, 1974) and repression of genes encoding heat shock proteins (Bissinger *et al.*, 1989; Pernambuco *et al.*, 1996) are two possible factors involved in the glucose-induced loss of stress resistance (Parsell *et al.*, 1993; Van Dijck *et al.*, 1995; Wiemken, 1990).

In this paper we describe a mutation in adenylate cyclase that causes a significant delay in glucose-induced mobilisation of trehalose and disappearance of heat shock protein messengers concomitant with a delay in glucose-induced loss of stress resistance. Since the mutation does not cause a delay in the initiation of growth and fermentation, it appears to offer a promising avenue for the development of industrial yeast strains with an improved maintenance of stress resistance during the initiation of fermentation.

## Results

### Isolation of the *fil1* Mutant

We have screened EMS-mutagenised yeast strains for mutants displaying a better maintenance of heat stress resistance during the initiation of fermentation. Three strains were used: the wild type strain M5 and the mutant strains *tps1 $\Delta$  hxx2 $\Delta$*  and *hxx2 $\Delta$*  in the same genetic background. The *tps1 $\Delta$*  mutant was used because it cannot synthesize trehalose. A *tps1 $\Delta$*  mutant, however, is unable to ferment and to grow on glucose because of deficient regulation of glucose influx into glycolysis (Thevelein and Hohmann, 1995). Hence, we have used the *tps1 $\Delta$  hxx2 $\Delta$*  strain, since the *hxx2 $\Delta$*  mutation suppresses this glucose negative phenotype (Hohmann *et al.*, 1993). The mutagenised cultures were grown to stationary phase and the cells were subsequently given 2% glucose to initiate fermentation. After 90 min in the presence of glucose a heat shock (30 min at 52°C) was given to kill cells that lost their heat resistance in a similar way as the non-mutagenised strain. To increase the stringency of the selection the same procedure was repeated with the surviving cells. A mutant (PVD1050) with the *hxx2 $\Delta$*  background showed the best maintenance of heat resistance during the initiation of fermentation and was selected for further characterisation. The mutation was called *fil1*, for 'deficient in fermentation-induced loss of stress resistance'.

### The Phenotype of the *fil1* Mutant is Due to a Single, Recessive Mutation

The original *fil1* mutant in the *hxx2 $\Delta$*  background (PVD1050) showed a remarkably high maintenance of heat resistance: after 30 min of fermentation it still displayed 100% survival upon heat shock whereas survival of the *hxx2 $\Delta$*  strain was less than 10% (Figure 1A). The *fil1 hxx2 $\Delta$*  mutant was crossed with the wild type strain M5 and the diploid strain showed the same rapid loss of heat resistance

as the isogenic diploid wild type strain, indicating that the *fil1* mutation is recessive (results not shown). Tetrad analysis of a *fil1/+ hxx2 $\Delta$ /+* diploid strain indicated a two-to-two segregation of the heat-stress resistant phenotype and lack of linkage between this phenotype and the *hxx2 $\Delta$*  mutation (results not shown). A segregant (PVD1150) with the heat-stress resistant phenotype but without the *hxx2 $\Delta$*  mutation was selected for further study. This *fil1* mutant showed a faster glucose-induced loss of heat resistance than the original double mutant *fil1 hxx2 $\Delta$* , while the *hxx2 $\Delta$*  mutant showed a somewhat better maintenance of heat resistance than the wild type strain (Figure 1A). Before and at all time points after addition of glucose the heat resistance of the *fil1* mutant was much higher than that of the wild type strain. Also the loss of heat resistance itself was slower in the *fil1* mutant than in the wild type strain. Before addition of glucose survival after heat shock was 10 times higher in the *fil1* mutant compared to the wild type strain while it was 100 times higher 90 min after addition of glucose (Figure 1A). The double mutant *fil1 hxx2 $\Delta$*  showed a remarkable maintenance of heat resistance after addition of glucose. Even after 90 min the survival percentage after a heat shock of 30 min at 52°C, that killed 99.9% of the cells of the wild type strain, was still 100% for the double mutant.

### The *fil1* Mutant Displays the Pleiotropic Phenotype of a cAMP Pathway Mutant

The *fil1* mutation also strongly affected the trehalose content of the cells. The initial level of trehalose, before addition of glucose to the cells, was 6-8 times higher in the two strains containing the *fil1* mutation (*fil1* and *fil1 hxx2 $\Delta$* ) compared to the control strains (wild type and *hxx2 $\Delta$* ) (Figure 1B). The latter two strains had a similar initial trehalose level and trehalose mobilisation after addition of glucose. Although the *fil1* mutant also displayed a rapid glucose-induced trehalose mobilisation, the trehalose content remained higher than in the wild type strain up to 90 min after addition of glucose. Up to 60 min after addition of glucose the trehalose content in the *fil1* mutant was at least as high as the initial level in the wild type strain. In the *fil1 hxx2 $\Delta$*  strain the effect on trehalose content was even more striking. Up to 90 min after addition of glucose virtually no net mobilisation of trehalose had occurred (Figure 1B). Since the *hxx2 $\Delta$*  strain showed a very similar trehalose mobilisation as the wild type strain, the combination of *fil1* and *hxx2 $\Delta$*  apparently results in a synergistic inhibitory effect on trehalose mobilisation.

Glucose-induced trehalase activation was reduced in the *fil1* mutant compared to the wild type strain and even more in the *fil1 hxx2 $\Delta$*  strain compared to the *hxx2 $\Delta$*  strain (Figure 1C), which correlates qualitatively with the effect on trehalose mobilisation (Figure 1B). However, the near-absence of trehalose mobilisation in the *fil1 hxx2 $\Delta$*  strain, at least up to 90 min after glucose addition, indicates that the activation of trehalase observed in that strain is either not sufficient to trigger trehalose mobilisation or that trehalose is also synthesised at the same time. Western blot experiments, using antibodies raised against the whole trehalose synthase complex, have revealed a tenfold overexpression of Tps1, Tps2 and Tsl1 in the *fil1* mutants (results not shown). This is consistent with the much higher trehalose level in the *fil1* mutants and the resulting high trehalose synthesising capacity might explain the absence

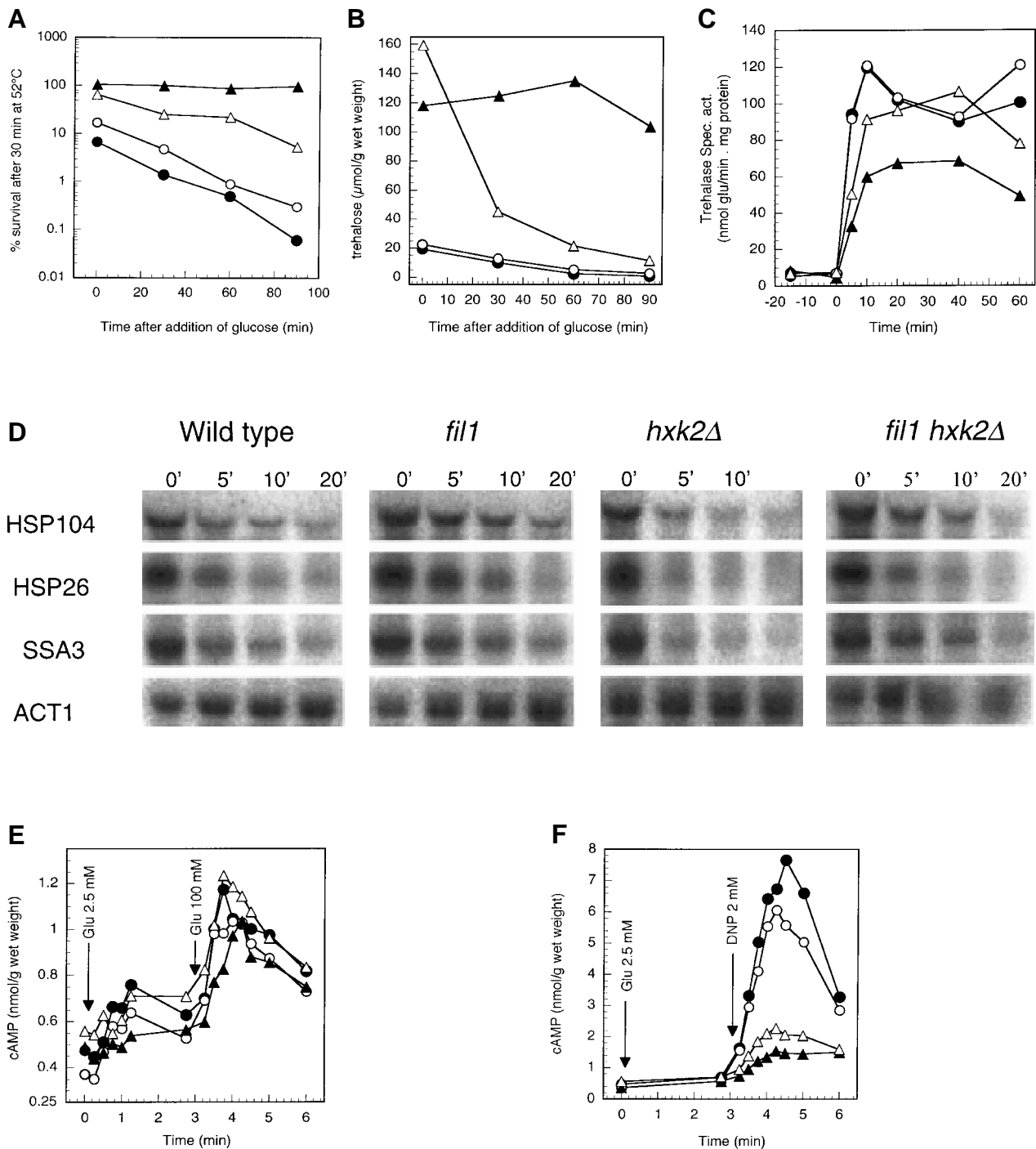


Figure 1. Effect of the *fil1* mutation on cAMP synthesis and cAMP pathway targets. A-E: glucose-induced effects. A. Loss of heat resistance. B. Trehalose mobilisation. C. Activation of trehalase. D. Repression of STRE-controlled Heat Shock Protein genes. E. cAMP increase. F. Intracellular-acidification induced cAMP increase (2,4-dinitrophenol at pH 6). Strains: wild type M5 (●), *hxx2Δ* (○), *fil1* (Δ) and *fil1 hxx2Δ* (▲).

of rapid glucose-induced trehalose mobilisation in spite of significant activation of trehalase.

Other factors known to be involved in conferring stress resistance in yeast are the heat shock proteins (Parsell *et al.*, 1993), in particular Hsp104 (Sanchez *et al.*, 1992). In the *fil1* mutant there was only a slight delay in the glucose-induced disappearance of the messengers of *HSP104*, *HSP26* and *SSA3* (encoding Hsp70) compared to the wild

type strain (Figure 1D). Also in the *fil1 hxx2Δ* strain repression of the STRE-controlled genes was only slightly delayed compared to the *hxx2Δ* control strain (Figure 1D). At time points between 30 and 180 min after addition of glucose there was no detectable difference anymore in the messenger levels (results not shown). This appears to indicate that still other factors play a role in the much higher maintenance of heat resistance in the two *fil1* mutant strains.

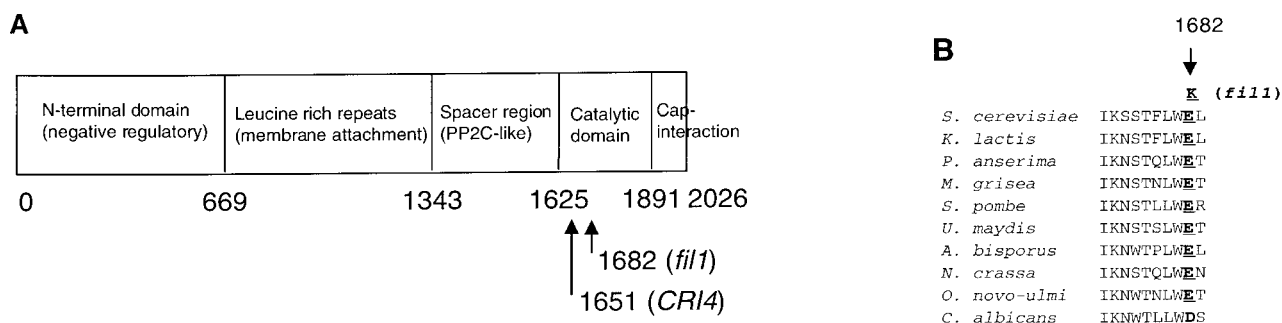


Figure 2. A. Scheme of functional and structural domains in *S. cerevisiae* adenylate cyclase. A large inhibitory domain is located in the N-terminal part of the enzyme. The much smaller catalytic domain is located at the C-terminus of the enzyme and is flanked by a small domain involved in binding of the Cap/Srv2 subunit at the extreme C-terminus. A large leucine-rich repeat domain and a spacer region which was shown to be required for Ras responsiveness are located in the middle of the enzyme. The *fil1* mutation (E1682K) is located in the beginning of the catalytic domain. B. Multiple alignment of fungal adenylate cyclases. Glutamate<sup>1682</sup> is conserved in all enzymes except in that of *Candida albicans* where this residue is changed into an aspartate, a conservative substitution.

We have also investigated whether the *fil1* mutation affected cAMP accumulation *in vivo*. For the glucose-induced cAMP signal we added 100 mM glucose 3 min after the addition of 2.5 mM glucose, to clearly separate the glucose-induced effect on cAMP synthesis from the effect of intracellular acidification (Colombo *et al.*, 1998). As shown in Figure 1E, the *fil1* mutant surprisingly displayed a very similar increase in cAMP after addition of 100 mM glucose compared to the wild type strain. Also in the *fil1 hxx2Δ* mutant there was only a slight reduction compared to the *hxx2Δ* strain. We also tested the increase in cAMP triggered by intracellular acidification. For that purpose 2 mM 2,4-dinitrophenol was added at an extracellular pH of 6 to cells of the four strains. Interestingly, in this case there was a strong reduction of the agonist-induced cAMP increase, pointing to a reduced cAMP accumulation capacity of the cells (Figure 1F). Closer examination of the cAMP increase triggered by 2.5 mM glucose, which is considered to be due to intracellular acidification (Colombo *et al.*, 1998), also shows a strong effect of the *fil1* mutation in both strains. When the glucose-induced cAMP signal was measured without pre-addition of a low glucose concentration, the signal was also reduced in the *fil1* mutant compared to the wild type strain (results not shown).

#### ***Fil1* is a Partially Inactivating Point Mutation in *CYR1*, the Gene Encoding Adenylate Cyclase**

We transformed the *fil1* mutant with the *CDC25*, *RAS2*, *CDC35* and *TPK1* genes each on a single copy plasmid. Expression of all these genes reduced to some extent the elevated heat resistance of the *fil1* mutant (results not shown). Subsequently, we transformed the *fil1* mutant with a single copy genomic library and screened for clones suppressing the heat-resistant phenotype. All genes isolated turned out to be suppressor genes rather than the *FIL1* gene. One of the suppressor genes was *SFI1*, an essential gene involved in mitosis (Ma *et al.*, 1999). Hence, we decided to identify the gene by mapping and chromosome walking. The *fil1* mutant was crossed with a set of mapping strains obtained from the Yeast Genetic Stock Center (see Materials and Methods) and the segregants were tested for linkage between the heat-stress resistant phenotype and the markers. The results indicated

that the *fil1* mutation was centromere linked on chromosome X (results not shown). Subsequently, five genomic sections of about ten kb each, located on the left arm and five located on the right arm of chromosome X next to the centromere, were amplified by PCR. The DNA fragments were expressed in the *fil1* mutant and the transformants tested for complementation of the heat resistant phenotype. Only one of the fragments caused complementation. It contained the *CYR1* gene, encoding adenylate cyclase, and the *CTK2* gene. Subcloning showed that only the *CYR1* gene complemented the *fil1* mutant (results not shown).

The precise location of the mutation in the *fil1* allele of *CYR1* was determined with the gap filling method (Iwasaki *et al.*, 1991). Different gaps were introduced in the wild type *CYR1* allele and filled by expression in the *fil1* mutant. Only one single fragment (*XhoI*-*Bst*EII) located in the 3' part of the gene, was unable to complement the *fil1* mutant (results not shown). Sequence analysis of this fragment either recovered by the gap filling method or PCR amplified using genomic DNA of the *fil1* mutant indicated that a single point mutation was present. It changed a glutamate residue into a lysine residue at position 1682 in the amino acid sequence.

Previously, several domains in yeast adenylate cyclase have been identified: a N-terminal inhibitory domain, a leucine-rich repeat domain, a domain required for Ras responsiveness and a C-terminal catalytic domain, flanked by a short domain required for interaction with the Cap/Srv2 subunit at the extreme C-terminus (Figure 2A) (Kataoka *et al.*, 1985; Masson *et al.*, 1986; Mintzer and Field, 1994; Uno *et al.*, 1987). However, the N- and C-termini also appear to contain residues required for proper Ras-dependent regulation of adenylate cyclase (Colicelli *et al.*, 1990; Field *et al.*, 1990; Heideman *et al.*, 1990; Suzuki *et al.*, 1990; Yamawaki-Kataoka *et al.*, 1989). Glutamate<sup>1682</sup> is located between the Ras-responsive domain of *S. cerevisiae* adenylate cyclase and the catalytic domain (Figure 2A). Comparative sequence analysis indicated that this glutamate residue is conserved in all fungal adenylate cyclases except that of *Candida albicans* where this residue is changed into an aspartate, a conservative substitution (Figure 2B). A mutation (*CRI4*) in a nearby residue, threonine1651, was isolated as a suppressor of a temperature-sensitive *ras1Δ ras2<sup>ts</sup>* mutant (De Vendittis

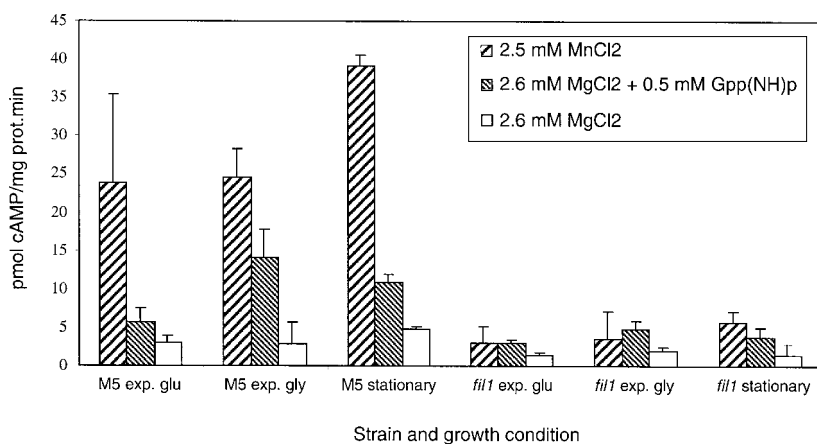


Figure 3. Adenylate cyclase activity in purified plasma membranes. The cells were grown on glucose into exponential phase or stationary phase and on glycerol into exponential phase. Enzyme activity was assayed in the presence of either manganese ions, magnesium ions or magnesium ions and the nonhydrolyzable GTP analogue guanosine-5'( $\beta$ , $\gamma$ -imino)triphosphate, GPP(NH)P. Strains: wild type M5, *fil1*.

*et al.*, 1986). Introduction of other mutations at the same site led to enhanced or attenuated activity depending on the new amino acid introduced (Feger *et al.*, 1991).

To investigate whether the *fil1* mutation affected catalytic activity of adenylate cyclase we have determined the activity of the enzyme in purified plasma membranes. The cells were grown on glucose into exponential phase or stationary phase and on glycerol into exponential phase. Adenylate cyclase activity was assayed in the presence of either manganese ions (G-protein independent activity), magnesium ions or magnesium ions and the nonhydrolyzable GTP analogue guanosine-5'( $\beta$ , $\gamma$ -imino)triphosphate, GPP(NH)P (Figure 3). In the three conditions enzyme activity was strongly reduced in the *fil1* mutant compared to the wild type strain and this was observed for all three growth conditions. The expression of the *CYR1* gene was also significantly enhanced in the *fil1* mutant at the mRNA level but not at the protein level (results not shown). In the *fil1* mutant the activity with magnesium and GPP(NH)P was similar to that with manganese. Both in the wild type strain and the *fil1* mutant the addition of GPP(NH)P caused a strong increase in adenylate cyclase activity. This shows that the *fil1* mutation does not affect the stimulation of adenylate cyclase by active G-protein but rather reduces intrinsic adenylate cyclase activity. Hence, the phenotype of the *fil1* mutant does not appear to be caused by a specific reduction in the responsiveness of adenylate cyclase to agonist stimulation, but rather by a lowering of the maximal activity that can be attained. This fits with the observation that the glucose-induced cAMP signal is not abolished in the *fil1* mutant but at most partially reduced.

#### The *fil1* Mutant has a Normal Growth and Fermentation Rate

Since mutants with reduced activity of the cAMP pathway are known to have a reduced growth rate and inactivation of the pathway causes permanent arrest in G<sub>0</sub>, we investigated whether the *fil1* mutant was compromised for growth rate and for fermentation activity. Whereas the original *fil1 hxx2* strain displayed a reduced growth rate and ethanol production compared to the wild type strain, the growth rate and ethanol production of the *fil1* mutant was not significantly different from that of the wild type strain (Figure 4). The final cell density of the *fil1* mutant was somewhat higher than that of the wild type strain and

ethanol reutilisation apparently somewhat slower (Figure 4). No growth defect on ethanol or other nonfermentable carbon sources, however, was observed (results not shown).

#### Introduction of the *fil1* Mutation in Other Genetic Backgrounds

To examine whether the *fil1* mutation could be used for the development of industrial baker's yeast strains with enhanced maintenance of stress resistance during the initiation of fermentation, we have introduced the mutation first in two other haploid laboratory strains with different genetic backgrounds, SP1 and W303-1A. The *fil1* mutants in the genetic backgrounds SP1, W303-1A and M5, displayed a similar increase in heat resistance in plate assays and also the maintenance of heat resistance during the initiation of fermentation was similar for the three strains (results not shown). The higher cell density was found in all genetic backgrounds tested. The cAMP signal was reduced with about 50% in the *fil1* mutants compared to the isogenic wild type strains (results not shown). Finally we also tested the desiccation resistance of the *fil1* mutant in two genetic backgrounds (M5 and SP1). For this purpose the residual fermentation capacity of the strains after drying was determined. The *fil1* mutants displayed a somewhat lower fermentation capacity before drying (Table 1). However, after drying and rehydration either at 20°C or 38°C the wild type strains rapidly lost fermentation capacity while the *fil1* mutants showed a much better maintenance of their fermentation capacity (Table 1).

#### Enhanced Stress Resistance in the Y55-*fil1* Strain After Production in Pilot Scale

To further evaluate the usefulness of the *fil1* mutation we have introduced it into a vigorously growing diploid laboratory strain, Y55, that can be cultivated in pilot scale under similar conditions as industrial yeast strains. The homozygous Y55-*fil1* mutant showed the same growth rate as the corresponding Y55 wild type strain and could also be grown under pilot scale at the same dilution rate as the Y55 wild type strain (results not shown). The yeast produced in this way was used for the preparation of frozen doughs. The frozen doughs were stored for different periods of time at -20°C, after which they were thawed and the gassing power measured. The results show that after prolonged storage the *fil1* mutant kept a higher fermentation

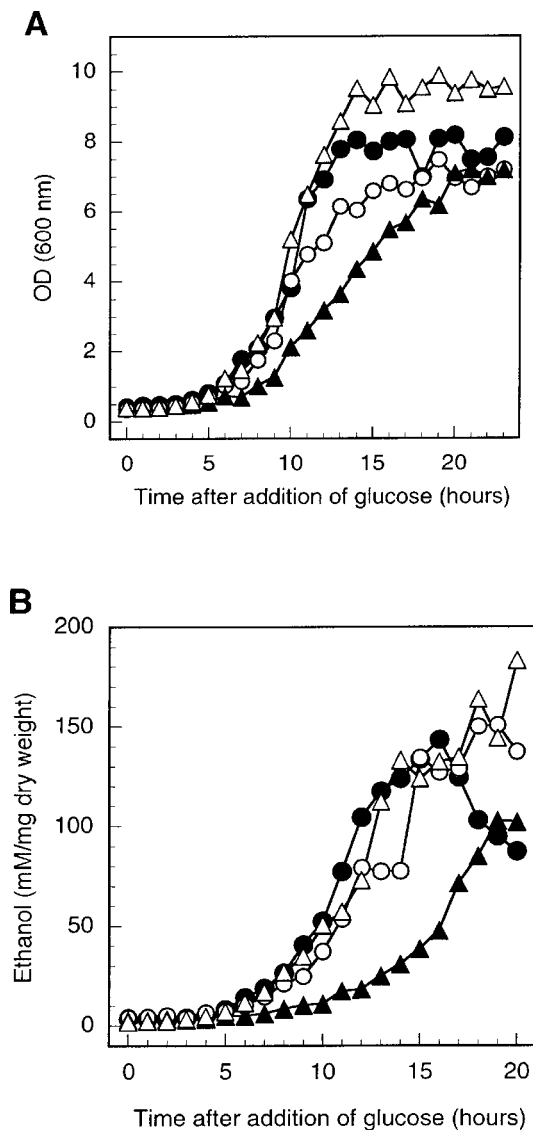


Figure 4. Growth (A) and ethanol production (B) after addition of glucose. Strains: wild type M5 (●), *hxxk2Δ* (○), *fil1* (Δ) and *fil1 hxxk2Δ* (▲).

capacity than the wild type strain (Figure 5A). This confirms the potential of the *fil1* mutation for the construction of industrial yeast strains with a better maintenance of freeze resistance. Similarly, when the yeast produced in pilot scale was tested for drying resistance, the *fil1* mutant showed a better retention of gassing power both in doughs with 1g and 4g sucrose (Figure 5B). This confirms that the *fil1* mutant in general displayed a higher stress resistance than the wild type strain.

## Discussion

***Fil1* is Allelic with *CYR1* Encoding Adenylate Cyclase**  
 Characterisation of the phenotype of the *fil1* mutant showed that it displayed the typical phenotype of a yeast mutant with reduced activity of the cAMP-PKA pathway. However, epistasis analysis with the main positively acting components of the pathway, *CDC25*, *RAS2*, *CYR1* and

*TPK1*, on single-copy plasmids indicated that they all to some extent suppressed the *fil1* phenotype. For a linear signaling pathway this would normally indicate that the *FIL1* gene product is located upstream of the first component, in this case *Cdc25*. The finding that the *fil1* mutation was allelic with the *CYR1* gene, encoding adenylate cyclase, was therefore rather unexpected. Moreover, inactivation of *CYR1* is lethal and partial inactivation of the cAMP-PKA pathway was known to reduce significantly the growth rate. The *fil1* mutation did not cause a growth problem. However, in view of the fact that the *fil1* mutation only partially inactivates adenylate cyclase it is understandable that enhanced expression of an upstream activating component can enhance the partial activity of the *fil1* allele of adenylate cyclase and therefore to some extent cause suppression of the *fil1* phenotype.

Intracellular acidification and glucose stimulate cAMP synthesis by two different mechanisms. Intracellular acidification activates the Ras proteins whereas the effect of glucose is mediated by the Gpr1-Gpa2 GPCR-like system (Thevelein and de Winde, 1999). The *fil1* mutation clearly had a much stronger reducing effect on acidification-induced cAMP accumulation than glucose-induced cAMP accumulation (Figure 1E,F). This further supports that the two phenomena involve different mechanisms. Since the extent of activation of adenylate cyclase *in vitro* by GPP(NH)P was not significantly affected by the *fil1* mutation, it appears that the Gpa2 protein might be more important for this activation *in vitro* than the Ras proteins. The observation that the *fil1* mutation affects glucose-activation of cAMP synthesis much less than acidification-induced activation makes it remarkable that the mutation was isolated in a screen for glucose-induced loss of stress resistance. It cannot be excluded, however, that the additional presence of the *hxxk2Δ* mutation has contributed significantly to the selective advantage of the original *fil1 hxxk2Δ* mutant strain isolated in the screen. In addition, it might indicate that under the normal conditions of an immediate glucose load, *i.e.* without pre-addition of a low glucose concentration, the intracellular acidification effect or at least the glucose phosphorylation dependent effect has a significant contribution in stimulation of the cAMP cascade.

The isolation of yeast mutants with a screening protocol based on glucose-induced loss of heat resistance appears to be a promising approach for elucidation of the glucose activation mechanism of the cAMP pathway. The finding that the *fil1* mutation is allelic with adenylate cyclase underscores the importance of the cAMP pathway for this

Table 1. Fermentation Capacity in *fil1* Mutants and Corresponding Wild Type Strains After Drying of the Yeast. (Residual fermentation capacity is also expressed as % of initial fermentation capacity.)

	M5 wild type	M5 <i>fil1</i>	SP1 wild type	SP1 <i>fil1</i>
Without drying	152 (100%)	127 (100%)	128 (100%)	116 (100%)
With drying and rehydration at 20°C	84.5 (55%)	101.5 (80%)	35 (37%)	78.5 (68%)
With drying and rehydration at 38°C	70 (46%)	109 (86%)	24 (19%)	67.5 (58%)

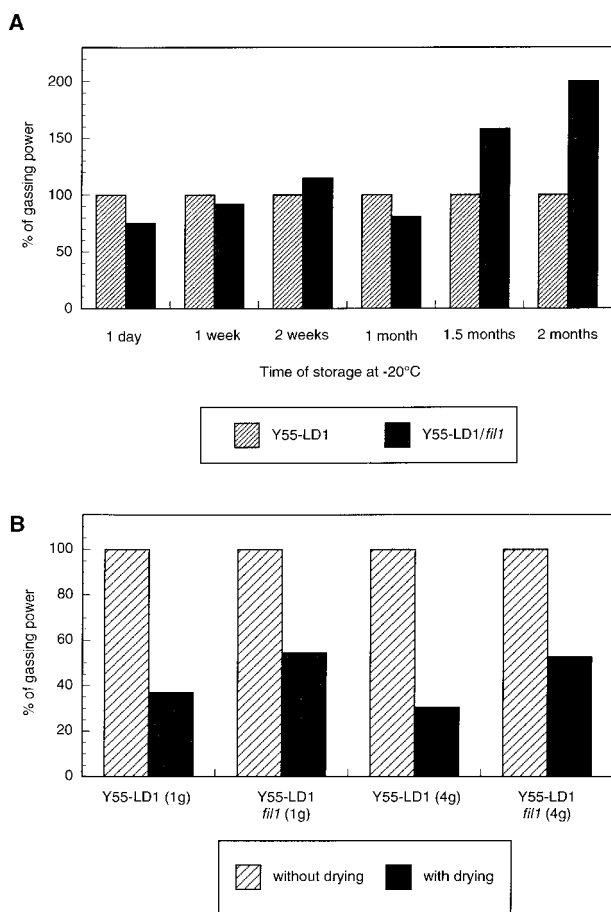


Figure 5. Gassing power (CO<sub>2</sub> produced during 2h at 30°C) in doughs prepared with yeast produced in pilot scale. A. Residual gassing power after freeze-storage of the doughs for different periods of time. The residual gassing power of the wild type strain Y55 is set at 100% in each case and compared with the residual gassing power of the *fil1* mutant in the same genetic background. B. Residual gassing power after drying and rehydration of the yeast before preparation of the doughs. The gassing power in doughs made with undried yeast was set each time at 100% and compared with the residual gassing power in doughs made with dried yeast. Doughs containing 1g or 4g sucrose were used.

phenomenon. Moreover, we have recently demonstrated that the *fil2* mutation is allelic with Gpr1, a putative glucose-sensing G-protein coupled receptor in the yeast plasma membrane (Kraakman *et al.*, 1999). A mutant in the G-protein, Gpa2, that interacts with Gpr1 also shows a delay in glucose-induced loss of heat resistance (Colombo *et al.*, 1998). Finally, a negative effector of glucose-activation of the cAMP pathway was recently isolated in a screen for multi-copy suppressors of glucose-induced loss of heat resistance (Versele *et al.*, 1999).

### Enhanced Stress Resistance is Compatible with Normal Growth and Metabolic Activity

In life forms in nature there is a striking inverse relationship between stress resistance and metabolic and proliferating activity. Well known examples are survival forms like spores, cysts and seeds, which display high stress

resistance and virtually no metabolic activity. Also in yeast cells there is a clear inverse correlation between stress resistance and growth and metabolic activity. Cells growing on fermentable carbon sources like glucose display the fastest growth rate and the lowest stress resistance while cells growing on nonfermentable carbon sources have a much slower growth rate and a higher stress resistance (Plesset *et al.*, 1987; Schenberg-Frascino and Moustacchi, 1972). Non-growing stationary phase cells and ascospores still display a somewhat higher stress resistance. When glucose is added to slow or non-growing cells they rapidly lose their stress resistance concomitant with the stimulation or induction of growth and fermentation (Van Dijck *et al.*, 1995). These observations suggest that there might be an incompatibility at the molecular level between high stress resistance and high metabolic activity. Hence, it could not be excluded that the aim of the baker's yeast industry, yeast strains displaying high stress resistance simultaneously with a high metabolic and proliferation activity, was 'against biological design' (Attfield, 1997).

Our results with the *fil1* mutant demonstrate that stress resistance can be enhanced at least to a certain extent without compromising growth and fermentation rate. The *fil1* mutant maintains to a certain extent the high heat resistance of stationary-phase cells during the initiation of growth and fermentation (Figure 1A). The original strain isolated in the screening procedure, *fil1 hxx2Δ*, even displayed an almost complete maintenance of heat resistance during the initiation of fermentation (Figure 1A). However, that strain was also compromised to a significant extent in the initiation of growth and ethanol production (Figure 4). Whether this indicates an incompatibility of high stress resistance and high metabolic activity or simply an involvement of the adenylate cyclase pathway in the start-up of growth and fermentation is not clear. The final cell density of the *fil1* mutant was somewhat elevated and ethanol reutilisation appeared somewhat slower. However, we could not detect any growth defect of the *fil1* mutant on ethanol or other nonfermentable carbon sources. Hence, it is unclear whether this could compromise the use of the strain in baker's yeast applications.

### The *hxx2Δ* Mutation Acts Synergistically with *fil1*

Although the *hxx2Δ* mutation has in itself only little effect on glucose-induced loss of heat resistance it causes a strong synergistic effect with the *fil1* mutation (Figure 1A). The same is true for glucose-induced mobilisation of trehalose and activation of trehalase. In these cases the *hxx2Δ* mutation has no effect at all but it causes a dramatic synergistic effect with the *fil1* mutation (Figure 1B, C). The best known physiological effect of a *hxx2Δ* mutation is inactivation of the main glucose repression pathway (Entian, 1980). Interestingly, an opposite effect has been observed previously on heat stress resistance of mutants in the cAMP pathway by the *snf1* mutation that causes inability of derepression (Thompson-Jaeger *et al.*, 1991). Hence, the rapid repression after addition of glucose of the targets of the main glucose repression pathway might be involved to a certain extent in glucose-induced loss of heat resistance. In wild type cells it appears to be overruled by glucose activation of the cAMP pathway since the *hxx2Δ* mutation had only little effect. However, when the activation of the cAMP pathway is reduced by the *fil1* mutation, the effect of the main glucose repression pathway becomes

Table 2. *S. cerevisiae* Strains Used in This Study. The wild type strain is a descendant of diploid strain M5 (Schaaff *et al.*, 1989).

Name	Relevant genotype	Complete genotype	Reference
YSH6.36-3B	wild type (M5 segregant)	Mat $\alpha$ <i>leu2-3, 112 ura3-52 trp1-92</i>	(Hohmann <i>et al.</i> , 1993)
YSH6.36-1C	wild type (M5 segregant)	Mata <i>leu2-3, 112 ura3-52 trp1-92</i>	(Hohmann <i>et al.</i> , 1993)
YSH317	<i>hvk2<math>\Delta</math></i>	Mat $\alpha$ <i>leu2-3, 112 ura3-52 trp1-92 hvk2<math>\Delta</math>::LEU2</i>	(Hohmann <i>et al.</i> , 1993)
YSH315	<i>tps1<math>\Delta</math>hvk2<math>\Delta</math></i>	Mat $\alpha$ <i>leu2-3, 112 ura3-52 trp1-92 hvk2<math>\Delta</math>::LEU2 tps1<math>\Delta</math>::TRP1</i>	(Hohmann <i>et al.</i> , 1993)
YSH292	<i>tps1<math>\Delta</math></i>	Mat $\alpha$ <i>leu2-3, 112 ura3-52 trp1-92 tps1<math>\Delta</math>::TRP1</i>	(Hohmann <i>et al.</i> , 1993)
PVD1050	<i>fil1hvk2<math>\Delta</math></i>	Mat $\alpha$ <i>leu2-3, 112 ura3-52 trp1-92 hvk2<math>\Delta</math>::Leu2 fil1</i>	This work
PVD1150	<i>fil1</i>	Mat $\alpha$ <i>leu2-3, 112 ura3-52 trp1-92 fil1</i>	This work
SP1	wild type	Mata <i>leu2 his3 trp1 ade8 can1 ura3</i>	
MV11	SP1 - <i>fil1</i>	Mata <i>leu2 his3 trp1 ade8 can1 ura3 fil1</i>	This work
Y55-LD1	prototrophic diploid strain derived from Y55	Mata/Mat $\alpha$	This work
Y55-LD4	Y55 - <i>fil1/fil1</i>	Mata/Mat $\alpha$ <i>fil1/fil1</i>	This work
S18-1D	<i>tpk1<sup>w1</sup> tpk2<math>\Delta</math> tpk3<math>\Delta</math></i>	Mat $\alpha$ <i>his3 leu2 ura3 trp1 ade8 tpk1<sup>w1</sup> tpk2::HIS3 tpk3::TRP1</i>	(Nikawa <i>et al.</i> , 1987)
RS13-58A-1	<i>tpk1<sup>w1</sup> tpk2<math>\Delta</math> tpk3<math>\Delta</math> bcy1<math>\Delta</math></i>	Mata <i>his3 leu2 ura3 trp1 ade8 tpk1<sup>w1</sup> tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	(Nikawa <i>et al.</i> , 1987)

much more important as evidenced by the synergistic effect with *hvk2 $\Delta$* .

### Better Maintenance of Stress Resistance During the Initiation of Fermentation

It is well known that industrially produced baker's yeast has an excellent stress resistance. Instant dry yeast can be stored at room temperature for up to 2 years without significant loss of activity. However, as soon as the yeast is used in dough making it initiates fermentation and rapidly loses stress resistance. This is detrimental for the preparation of doughs in which the yeast experiences high stress, such as in freeze doughs (Attfield, 1997). The main challenge to address this problem is to identify mutations that allow a better maintenance of stress resistance during the initiation of fermentation without compromising the start-up of growth and fermentation. In this paper we show that the *fil1* mutation, at least in a laboratory strain background, has this effect. Also after growth in pilot scale under similar growth conditions as for industrial baker's yeast the Y55-*fil1* strain displayed a better freezing and drying resistance than the Y55 control strain (Figure 5). If a *fil1* industrial strain would display the same behaviour as the laboratory *fil1* mutant it would present a considerable advantage compared to the industrial yeast strains now in use.

### Experimental Procedures

#### Yeast Strains

*S. cerevisiae* strains used in this study are shown in Table 2. The Y55-LD1 diploid strain was constructed as follows. The strains Y55-2160 (Mat $\alpha$  *trp1-Bsu361 ade1-1 ho $\Delta$* ) and Y55-2202 (Mata *ura3-Nco1 leu2 ho $\Delta$* ), kindly provided by Dr. Louis Edward (Oxford, U.K.), were crossed and a prototrophic segregant was isolated by tetrad analysis. The mating type of this strain was changed by introduction of a plasmid with the HO gene and a completely homozygous diploid strain was constructed by crossing the two strains of opposite mating type. The resulting strain was called Y55-LD1. The *fil1* mutants were constructed as detailed below.

Mapping strains obtained from the Yeast Genetic Stock Center were: X4119-19C, X4119-15D, STX145-13D, STX145-15D, STX66-4A, STX82-3A, STX146-19A, STX153-10C, STX84-5A, STX75-3C, STX147-9B, STX147-4C, STX83-17D, X4120-19D, X4126-6D, STX77-6C, STX155-3C and STX155-9B.

### Plasmid Constructions and Gap Filling Method

The full length *CYR1* gene was isolated from a genomic library clone (Vanhalewyn *et al.*, 1999) and inserted in the vector YCplac33 (Gietz and Sugino, 1988). This plasmid was used for complementation studies with the *fil1* mutants and for the isolation of the *fil1* allele using the gap-filling method (Iwasaki *et al.*, 1991). A *Pst*I-*Bam*HI fragment (2794 bp) containing the 3' part of *CYR1*, including the *fil1* mutation, was subcloned in pUC18. The *URA3* marker (isolated from plasmid pJJ242, (Jones and Prakash, 1990) was subcloned into the unique *Sna*BI site, located in the 3' noncoding part of *CYR1*. This new construct, pMV13, was used to introduce the *fil1* mutation into the SP1 strain. For this purpose pMV13 was digested with *Msc*I and transformed into a *ura3* strain. Correct introduction of the *fil1* mutation was each time checked by PCR and sequence analysis. For introduction of the *fil1* mutation into the prototrophic Y55-LD1 strain, the *URA3* marker in the construct was exchanged for the dominant genetic marker and the construct was used to create a diploid, heterozygous *fil1/FIL1* strain. After sporulation two haploid *fil1* segregants were crossed to obtain a diploid, homozygous *fil1/fil1* strain.

### EMS Mutagenesis and Selection of Mutants

Ethylmethanesulfonate (EMS) mutagenesis was performed according to standard procedures (Sherman *et al.*, 1986). After EMS treatment, the mutagenised cells were washed, resuspended in YPD (1% yeast extract, 2% bacto peptone, 2% glucose) and grown till stationary phase. An aliquot of 0.5 ml of this culture was transferred into 25 ml fresh YPD and incubated at 30°C for 90 min. The culture was then heated in a water bath at 52°C for 30 min and subsequently further incubated at 30°C. When the cells had grown again into stationary phase 0.5 ml of the culture was transferred to a new flask with 25 ml YPD and incubated at 30°C for 90 min. Subsequently 100  $\mu$ l aliquots of the culture were heat shocked at 52°C, 54°C or 56°C. The colonies recovered from the 56°C heat shock included the *fil1* mutant.

### Culture and Incubation Conditions

Yeast cells were grown in YPD medium at 30°C to stationary phase in shake flasks in a gyratory incubator. The cells were harvested, washed and resuspended in YP medium. Subsequently they were preincubated for 45 min at 30°C after which glucose was added to a final concentration of 100 mM. Samples were taken just before and at 30, 60 and 90 min after addition of glucose.

### Growth Curves, Glucose Consumption and Ethanol Production

Yeast cells were inoculated in YPD medium and growth was followed for 24 hours. Samples were taken every hour to measure the OD<sub>600</sub> and to determine glucose and ethanol concentrations in the medium. Glucose levels were determined by the glucose oxidase/peroxidase method. Ethanol levels were determined using alcohol oxidase (kindly provided by Dr. J.P. van Dijken, Delft, the Netherlands) as described by Verduyn *et al.* (1984). Growth under pilot scale conditions was performed as described previously (Van Dijck *et al.*, 1995).

### Heat and Freeze Resistance

For determination of heat and freeze resistance, the cells were washed with ice-cold water and resuspended in ice-cold YPGlycerol. After dilution with the same medium to an optical density of 1 at 600 nm, 0.5 ml of the cell suspension was either incubated at 52°C for 15 or 30 min for determination of heat resistance or stored at -20°C for 5 or 12 days for determination of freeze resistance. Non-treated cells served as a control. After heat shock, the cells were quickly cooled on ice and then diluted 10<sup>3</sup> and 10<sup>4</sup> times. A sample (0.1 ml) of each dilution was spread on YPD plates or on YPGlycerol plates in the case of the *tps1Δ* or *tps1Δ hxx2Δ* strains. Survival after heat shock or freeze treatment was estimated by counting the number of colonies after 2 days of growth at 30°C. Survival rates are expressed as percentages of the number of colonies relative to the number of colonies in the non-stressed control samples.

### Heat Tests on Plate

Freshly streaked yeast strains were grown overnight on plates containing 2% glucose (master plates). The master plates were rapidly replicated onto fresh plates preheated at 56°C for at least one hour. Usually three replicas were made which were then incubated for different time periods at 56°C. After the heat shock the plates were incubated for 3 days at 30°C and photographed.

### Freeze Resistance Tests in Doughs

Yeast cells were cultivated on molasses, in a fed batch mode in pilot scale conditions as described previously (Van Dijck *et al.*, 1995). They were used to prepare doughs as follows, 160 mg (dry weight equivalent) of yeast were suspended in 15 ml of water containing 27 g/l of NaCl and 4 g/l of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and further mixed with 20 g of flour and 1 g of sucrose. The doughs obtained (6 per strain) were immediately incubated at 30°C for 30 minutes and then stored at -20°C for periods of 1 day up to 2 months. The fermentation capacity after storage at -20°C was measured by incubating the frozen dough at 30°C for 13 minutes, then sealing hermetically the vessel containing the dough and measuring the total quantity of CO<sub>2</sub> produced after 2 and 4 hours using the fermentometer of Burrows and Harrison (1959; Godon and Loisel, 1984). The quantity of CO<sub>2</sub> produced under 760 mm of mercury (Hg) is expressed in ml at 20°C.

The residual CO<sub>2</sub> production of the dough made with the control strain is used for each storage period as reference (100%). The stability upon freezing is then expressed as the ratio of CO<sub>2</sub> released during 2 hours after a certain period of storage at -20°C and the reference value.

### Dessication Test

Yeast cells are cultivated on molasses, in fed batch pilot scale conditions as described previously (Van Dijck *et al.*, 1995). Cells are further dried using a process of fluidization type (Clément and Loiez, 1983). An aliquot of non-dried cells is stored to be used as reference. Dough is prepared by mixing 160 mg (dry weight) of yeast cells previously resuspended in 15 ml of water containing NaCl (27g/l) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (4 g/l) to 20 g of flour and 1 g of sucrose. Dried yeast cells are rehydrated for 15 minutes in water, at 20°C or 38°C before mixing. Doughs are incubated at 30°C for thirteen minutes then the vessels containing the doughs are hermetically sealed and the carbon dioxide production is measured after 2 and 4 hours using a fermentometer (Burrows and Harrison, 1959).

### Western Blot Experiments

Crude membranes were prepared as described for the adenylate cyclase measurements. 2.5 μg of crude membranes were loaded on a 7.5% SDS PAGE gel. After blotting, the nitrocellulose membranes were incubated for 1 h in TBST (25 mM Tris/HCl pH 8, 150 mM NaCl, 0.05 vol% Tween 20) containing 5% non-fat dry milk. Then the primary antibody (1:100 dilution of CDC35(yC-20), Santa Cruz Biotechnology, #sc-6763) was added in the same solution and incubated overnight at 4°C. After washing in TBST, the secondary antibody (1:5000 of HRP-Rabbit anti-Goat IgG, Zymed Laboratories) was added in TBST containing 5% non-fat dry milk and the membranes incubated further for 45 min. After washing, detection was performed essentially as described in the ECL+ detection kit of Amersham.

### Biochemical Determinations

Trehalose levels were determined using *Humicola grisea* trehalase, glucose oxidase and peroxidase (Neves *et al.*, 1994). For determination of the cAMP level the cells were grown on YPGlycerol until exponential phase, harvested and resuspended in 25 mM Mes buffer (pH 6). For determination of glucose-induced cAMP accumulation, they were preincubated for 10 min before addition of 5 mM glucose, followed after 5 min by addition of 100 mM glucose. For determination of acidification-induced cAMP accumulation the cells were preincubated for 10 min before addition of 2 mM 2,4-dinitrophenol. Extraction of the cells and determination of the cAMP level was performed as described previously (Thevelein *et al.*, 1987). Trehalase activity was

determined in crude cell extracts as described previously (Pernambuco *et al.*, 1996).

### Adenylate Cyclase Assay

Preparation of crude yeast membranes for the adenylate cyclase assay was performed essentially as described by (Mintzer and Field, 1995). Adenylate cyclase activity was assayed in a reaction mixture containing 20 mM Mes pH 6.2, 0.1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 0.25 mM IBMX, 0.1 mg/ml BSA, 0.25 mM cAMP, 1 mM [α-<sup>32</sup>P]ATP (final spec. act. 30-100 cpm/pmol), 20 mM phosphocreatine, 0.25 mg/ml creatine phosphokinase, 0.1 mM DTT, either in the presence of 2.5 mM MnCl<sub>2</sub>, 2.5 mM MgCl<sub>2</sub> or 2.5 mM MgCl<sub>2</sub> and 500 μM Gpp(NH)p. The reaction was initiated by addition of the assay mixture to the membranes (10-80 μg of protein) in a final volume of 100 μl. The tubes were immediately transferred to a 30°C water bath and incubated for 20 min. The reaction was stopped by addition of 0.8 ml of 'stopping solution' containing 10 mM Tris/HCl, pH 7.5, 0.175 mM cAMP, 5 mM ATP and 0.25% sodium dodecylsulfate. [<sup>3</sup>H]cAMP (approximately 20,000 cpm) in 100 μl was added to monitor sample recovery. <sup>32</sup>P-cAMP produced was determined essentially as described by (Solomon *et al.*, 1973).

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