

# A mutation in *Saccharomyces cerevisiae* adenylate cyclase, $\text{Cyr1}^{\text{K1876M}}$ , specifically affects glucose- and acidification-induced cAMP signalling and not the basal cAMP level

Mieke Vanhalewyn,<sup>1</sup> Françoise Dumortier,<sup>1</sup> Gilda Debast,<sup>1</sup> Sonia Colombo,<sup>1,2</sup> Pingsheng Ma,<sup>1</sup> Joris Winderickx,<sup>1</sup> Patrick Van Dijck<sup>1,2</sup> and Johan M. Thevelein<sup>1\*</sup>

<sup>1</sup>Laboratorium voor Moleculaire Celbiologie, Institute of Botany and Microbiology, Katholieke Universiteit Leuven and <sup>2</sup>Flanders Interuniversity Institute for Biotechnology – VIB, Kardinaal Mercierlaan 92, B-3001 Leuven-Heverlee, Flanders, Belgium.

## Summary

In the yeast *Saccharomyces cerevisiae*, the addition of glucose to derepressed cells and intracellular acidification trigger a rapid increase in the cAMP level within 1 min. We have identified a mutation in the genetic background of several related 'wild-type' laboratory yeast strains (e.g. ENY.cat80-7A, CEN.PK2-1C) that largely prevents both cAMP responses, and we have called it *lcr1* (for lack of cAMP responses). Subsequent analysis showed that *lcr1* was allelic to *CYR1/CDC35*, encoding adenylate cyclase, and that it contained an A to T substitution at position 5627. This corresponds to a K1876M substitution near the end of the catalytic domain in adenylate cyclase. Introduction of the A5627T mutation into the *CYR1* gene of a W303-1A wild-type strain largely eliminated glucose- and acidification-induced cAMP signalling and also the transient cAMP increase that occurs in the lag phase of growth. Hence, lysine<sup>1876</sup> of adenylate cyclase is essential for cAMP responses *in vivo*. Lysine<sup>1876</sup> is conserved in *Schizosaccharomyces pombe* adenylate cyclase.  $\text{Mn}^{2+}$ -dependent adenylate cyclase activity in isolated plasma membranes of the  $\text{cyr1}^{\text{met1876}}$  (*lcr1*) strain was similar to that in the isogenic wild-type strain, but  $\text{GTP/Mg}^{2+}$ -dependent activity was strongly reduced, consistent with the absence of signalling through adenylate cyclase *in vivo*. Glucose-induced activation of trehalase was reduced and mobilization of trehalose and glycogen

and loss of stress resistance were delayed in the  $\text{cyr1}^{\text{met1876}}$  (*lcr1*) mutant. During exponential growth on glucose, there was little effect on these protein kinase A (PKA) targets, indicating that the importance of glucose-induced cAMP signalling is restricted to the transition from gluconeogenic/respiratory to fermentative growth. Inhibition of growth by weak acids was reduced, consistent with prevention of the intracellular acidification effect on cAMP by the  $\text{cyr1}^{\text{met1876}}$  (*lcr1*) mutation. The mutation partially suppressed the effect of  $\text{RAS2}^{\text{val19}}$  and  $\text{GPA2}^{\text{val132}}$  on several PKA targets. These results demonstrate the usefulness of the  $\text{cyr1}^{\text{met1876}}$  (*lcr1*) mutation for epistasis studies on the signalling function of the cAMP pathway.

## Introduction

As in other eukaryotes, cAMP is synthesised by the enzyme adenylate cyclase in the yeast *Saccharomyces cerevisiae*. It is encoded by the *CYR1/CDC35* gene (Matsumoto *et al.*, 1984). The enzyme is 2026 amino acids long and is composed of an N-terminal inhibitory domain, a leucine-rich repeat domain and a C-terminal catalytic domain (Kataoka *et al.*, 1985). It belongs to a family of adenylate and guanylate cyclases with widespread occurrence (Danchin, 1993). Adenylate cyclase activity in isolated plasma membranes of *S. cerevisiae* cells is largely GTP independent when measured in the presence of  $\text{Mn}^{2+}$ . When measured in the presence of  $\text{Mg}^{2+}$ , the activity is much lower but can be stimulated severalfold by the addition of GppNHp, reflecting  $\text{GTP/Mg}^{2+}$ -dependent activity (Varimo and Londesborough, 1976).

Extensive biochemical and genetic evidence has shown that the yeast Ras1 and Ras2 proteins are controlling elements of adenylate cyclase and that, in their absence, cAMP synthesis *in vivo* is insufficient for viability (Toda *et al.*, 1985; Field *et al.*, 1988). It has been proposed that the Ras proteins interact with the N-terminal inhibitory domain of adenylate cyclase to relieve its inhibition on the catalytic domain (Heideman *et al.*, 1987). Other results have implicated the C-terminal last 100 amino acid residues of adenylate cyclase (Yamawaki-Kataoka *et al.*,

Received 22 February, 1999; revised 18 April, 1999; accepted 23 April, 1999. \*For correspondence. E-mail johan.thevelein@bio.kuleuven.ac.be; Tel. (+32) 16 321507; Fax (+32) 16 321979.

1989) and a domain N-terminally adjacent to the catalytic domain (Uno *et al.*, 1987) as important for interaction with Ras. The activity of the Ras proteins is controlled by the guanine nucleotide exchange factor Cdc25 (Camonis *et al.*, 1986) and the GTPase activating proteins Ira1 (Tanaka *et al.*, 1989) and Ira2 (Tanaka *et al.*, 1990). cAMP controls the activity of protein kinase A (PKA), of which the catalytic subunits are encoded by the *TPK1*, *TPK2* and *TPK3* genes (Toda *et al.*, 1987a), and the regulatory subunits by the *BCY1* gene (Toda *et al.*, 1987b).

Biochemical and genetic work has identified several targets of the cAMP pathway. High activity of the pathway causes low levels of trehalose and glycogen, low heat resistance and a reduced expression level of 'STRE'-controlled genes, such as *CTT1* (catalase) and *SSA3* (Hsp70). Low activity of the pathway causes the opposite phenotype (for reviews, see Broach and Deschenes, 1990; Thevelein, 1992; 1994; Tatchell, 1993; Ruis and Schuller, 1995). This is particularly evident during exponential growth on glucose, as trehalose and glycogen levels, heat resistance and the expression of 'STRE'-controlled genes are low in this condition in wild-type cells. There is considerable evidence that these PKA targets are also controlled by one or more cAMP-independent pathways (Belazzi *et al.*, 1991; Hirimburegama *et al.*, 1992; Durnez *et al.*, 1994; Pernambuco *et al.*, 1996; Crauwels *et al.*, 1997). Whether PKA is directly involved in this alternative regulation is not clear. Several biochemical targets of PKA control have been identified in yeast, e.g. trehalase (Uno *et al.*, 1983), fructose-1,6-bisphosphatase (Müller and Holzer, 1981), glycogen phosphorylase (Lin *et al.*, 1995) and phosphatidylserine synthase (Kinney and Carman, 1988).

Only two triggers of the cAMP pathway are well established in yeast (Thevelein, 1991). The addition of glucose to derepressed yeast cells, but not to glucose-repressed cells, triggers a transient increase in the cAMP level within 1 min (van der Plaats, 1974). Intracellular acidification triggers an equally rapid but more pronounced and longer lasting increase in the cAMP level (Caspani *et al.*, 1985). Glucose does not act through an intracellular acidification effect (Thevelein *et al.*, 1987a). Both phenomena of cAMP signalling are specifically controlled by the low-affinity Pde1 phosphodiesterase, as opposed to the basal cAMP level in the cells, which is specifically controlled by the high-affinity Pde2 phosphodiesterase (Ma *et al.*, 1999). Recent work has also identified a transient lag phase-associated increase in the basal cAMP level (Ma *et al.*, 1997).

Although genetic evidence indicates an involvement of the Ras proteins as signal transmitters for glucose- and intracellular acidification-induced cAMP signalling (Mbonyi *et al.*, 1988), recent work has shown that only intracellular acidification enhances the GTP content on the Ras

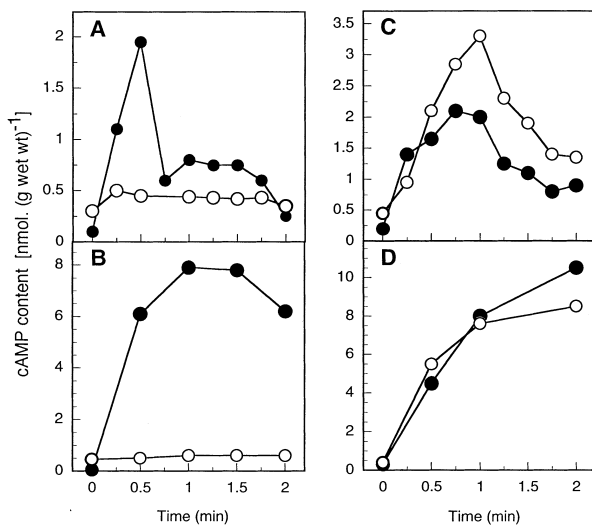
proteins. The addition of glucose does not affect the ratio of GTP/GDP bound to the Ras proteins (Colombo *et al.*, 1998). On the other hand, the G-protein encoded by the *GPA2* gene (Nakafuku *et al.*, 1988) is required for glucose-induced but not for acidification-induced cAMP signalling (Colombo *et al.*, 1998). Recent work has also identified a protein, called Gpr1, with structural and functional homology to G-protein-coupled receptors, of which the C-terminus binds to Gpa2. Gpr1 is also required for glucose-induced activation of cAMP synthesis, suggesting that glucose sensing for activation of the cAMP pathway might be carried out by a G-protein-coupled receptor system consisting of Gpr1-Gpa2 (Kraakman *et al.*, 1999).

During our studies of the glucose-induced cAMP signal in glucose repression mutants (Argüelles *et al.*, 1990) and in hexokinase and glucokinase deletion mutants in the same genetic background, we have discovered that, in this background, a mutation was present that nearly abolished both glucose- and acidification-induced cAMP signalling. These strains were kindly provided by K. D. Entian (Frankfurt, Germany). The corresponding wild-type strains containing this mutation had been used for many years as wild-type laboratory strains, and one of these was used to construct the CEN.PK series of wild-type strains (Randez-Gil *et al.*, 1997). In the present work, we have identified this mutation as a K1876M substitution in adenylate cyclase. We show that the mutation does not reduce the maximal  $Mn^{2+}$ -dependent activity of the enzyme *in vitro*, but rather affects its  $GTP/Mg^{2+}$ -dependent activation. *In vivo*, the K1876M mutation in adenylate cyclase affects all PKA targets investigated, mainly during the transition from gluconeogenic/respirative to fermentative growth.

## Results

### Discovery and segregation of the mutation

The addition of glucose to derepressed yeast cells triggers a rapid, transient increase in the cAMP level within a few minutes (van der Plaats, 1974), while intracellular acidification, for instance by the addition of protonophores, causes a much higher and longer lasting cAMP increase (Caspani *et al.*, 1985). For our studies of the mechanisms underlying these agonist-induced cAMP increases, several mutant strains have kindly been provided by K. D. Entian (Frankfurt, Germany). Remarkably, all strains derived from a common genetic origin, including the 'wild-type' derivatives, lacked the cAMP increase induced by both glucose and the protonophore 2,4-dinitrophenol. This is shown in Fig. 1A and B for the 'wild-type' derivative ENY.cat80-7A. In the same figure, the glucose- and acidification-induced cAMP responses in the wild-type strain SP1 are shown for comparison. The mutation is apparently also present in a



**Fig. 1.** Intracellular cAMP level as a function of time after the addition of 100 mM glucose (A and C) or 2 mM 2,4-dinitrophenol (B and D). A and B. Wild-type strain SP1 (●), *lcr1* mutant strain ENY.cat80-7A (○). C and D. *lcr1* + YCpCYR1 (MV7159) (●), *LCR1* + YCpCYR1 (MV7161) (○).

new standard laboratory strain, CEN.PK2-1C, that has been derived from this background (Randez-Gil *et al.*, 1997). This strain also lacks glucose- and acidification-induced cAMP responses (results not shown).

Two strains lacking the cAMP responses, ENY.cat80-7A and ENY.cat80-8A were crossed with the SP1 wild-type strain. The diploid strains all showed normal cAMP responses, indicating that the mutation(s) involved were recessive (results not shown). Tetrad analysis of one of the diploid strains showed, for four asci, a two-to-two segregation of the mutant and wild-type phenotype, indicating that only one mutation was involved (results not shown). The glucose- and acidification-induced cAMP responses co-segregated. The mutation causing absence of the cAMP responses was called *lcr1*, for 'lack of cAMP responses'.

#### Genomic mapping of the mutation

Further analysis of the phenotype of the *lcr1* strains revealed that they displayed a reduced rate of 2,4-dinitrophenol-induced trehalose mobilization (results not shown). However, using this phenotype in a microtitre plate-based screen, we were unable to isolate a complementing clone from a yeast genomic library. Therefore, we decided to map the mutation in order to associate it with a known gene or to use chromosome walking in case of an unknown gene. An *lcr1* mutant strain was crossed with a set of mapping strains obtained from the Yeast Genetic Stock Center (see *Experimental procedures*). Measurement

of the 2,4-dinitrophenol-induced cAMP response in the segregants indicated a clear two-to-two segregation in all cases. Moreover, in all crosses in which a centromere-linked marker gene (*ade1*, *trp1*, *leu1*, *leu2*, *pet8*) was involved, the number of tetratype tetrads was reduced, indicating centromere linkage of the *lcr1* mutation. In addition, the results of a cross with an *ilv3*-containing strain indicated linkage between *lcr1* and *ilv3* (10 cM). The *ILV3* gene is located on chromosome X, and the *CYR1* gene, encoding adenylate cyclase, is located close to the centromere of chromosome X. Therefore, we investigated whether *lcr1* could be allelic with *CYR1*.

#### The *lcr1* mutation is allelic with *CYR1*

We crossed an *lcr1* strain with a strain containing the temperature-sensitive *cdc35-10* allele and examined the progeny for absence of cAMP signalling (presence of *lcr1*) and for thermosensitive growth (presence of *cdc35-10*). All 10 tetrads investigated were parental ditype, indicating allelism or very close linkage. Further support that *lcr1* and *CYR1* were allelic was obtained by complementation of an *lcr1* mutant (strain MV7117C) with a plasmid containing the wild-type *CYR1* gene. With both an episomal plasmid (pYACE1) (results not shown) and a centromeric plasmid (YCplac33YACE1) (Fig. 1C and D) containing the wild-type *CYR1* gene, the glucose- and acidification-induced cAMP responses were restored. Subsequently, we measured the cAMP responses in a diploid *lcr1/cdc35-10* strain (MV7143) at the permissive (24°C) and restrictive (37°C) temperatures for the *cdc35-10* mutation. At 37°C, cAMP signalling was largely absent compared with 24°C, indicating that *lcr1* was allelic with *CYR1* (results not shown).

#### A *Ty* element in the promoter of the *lcr1* allele is not responsible for the absence of the cAMP responses

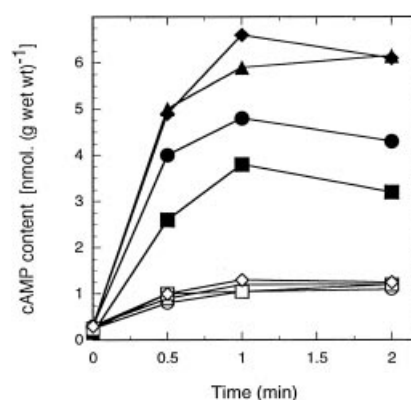
Southern blot analysis with *EcoRI*-digested DNA of the four strains in a tetrad (strains MV7117A–D) showed that the *lcr1* descendants had a different restriction pattern compared with the *CYR1* descendants. Instead of a single 3.5 kb band in the wild-type strains, two bands of 3 kb and 8 kb were observed (using a 2.6 kb *SphI*–*EcoRI* probe, covering part of the promoter and a 405 bp-long initial part of the wild-type *CYR1* gene). Southern blot analysis of the parental strains (SP1, ENY.cat80-7A and ENY.cat80-8A) and the descendants of another tetrad (strains MV7106A–D) confirmed that this aberrant restriction pattern was linked with the *lcr1* mutation (results not shown). Based on this result and on additional results obtained with other restriction enzymes and other probes (not shown), we concluded that an insertion of unknown length was present 5' upstream of the *PvuII* site (position –142) in the promoter of the *lcr1* mutant allele. We cloned

the promoter with the unknown insert from an *lcr1* mutant strain using plasmid eviction (see *Experimental procedures*). Sequence analysis showed that the insertion was a Ty transposable element (Boeke and Sandmeyer, 1991) and that it ended at position -555 in the promoter of the *lcr1* mutant allele (results not shown). Two previous reports have dealt with the insertion of a Ty element into the promoter and into the regions -4 to +82 and +124 to +268 of the *CYR1* gene (Iida, 1988; Lenzen *et al.*, 1987, respectively). In the latter case, it caused a strong reduction in adenylate cyclase activity, slow growth and a multistress-resistant phenotype.

To check whether the presence of the Ty element in the promoter was responsible for the absence of the cAMP responses, for instance by reducing the transcription of the *CYR1* gene, we inserted an integrating plasmid of the Ylplac series (see *Experimental procedures*) at different positions upstream of the start codon. Insertion at positions -1790, -1590, -840 and -526 (results not shown) did not affect the acidification-induced cAMP increase. As the Ty transposon ended at position -555, this made it unlikely that it was responsible for the absence of the cAMP responses.

*Construction of chimeric genes indicates that a mutation located in the 3'-third of the lcr1 allele is responsible for the phenotype*

To investigate which part of the *lcr1* mutant allele was responsible for the absence of the cAMP responses, we constructed chimeric genes consisting of three different fragments derived from the wild-type *CYR1* gene (designated W for wild type) or the *lcr1* mutant allele (designated M for mutant). The fragments (positions in brackets) were *SphI* (in MCS of pUC19\*)-*Asp718* (120), *Asp718* (120)-*NcoI* (4823) and *NcoI* (4823)-*BamHI* (6534). The first fragment contained about 1.3 kb of the promoter, including part of the Ty transposon in the case of the *lcr1* mutant gene. The last fragment contained about 0.45 kb of the terminator. (For the construction of these plasmids, see *Experimental procedures*.) The eight possible combinations of chimeric genes were introduced on a centromeric plasmid (designated pWWW, pMMM, pWWM, pMWW, pMWM, pWMM, pMWM, pMMW) into an *lcr1* strain (MV7117C). Measurement of the 2,4-dinitrophenol-induced cAMP increase in these transformants clearly indicated that the mutation responsible for the absence of the cAMP responses was located in the C-terminal part of the protein (Fig. 2). Moreover, the presence of the 5' *lcr1* fragment, which contained part of the Ty transposon, also resulted in normal cAMP responses when a wild-type 3'-terminal fragment was present. This confirmed that the Ty transposon was not responsible for the absence of the cAMP responses.



**Fig. 2.** Intracellular cAMP level after the addition of 2,4-dinitrophenol to cells of an *lcr1* strain (MV7117C) transformed with the following plasmids: pWWW (●), pWMM (▲), pMWW (■), pMMW (◆) (each restoring a normal cAMP increase) and pWWW (○), pWMM (△), pMWM (□), pMMM (◇) (each not restoring the cAMP increase). W, first, second or third part of the wild-type *CYR1* gene; M, first, second or third part of the *lcr1* mutant allele.

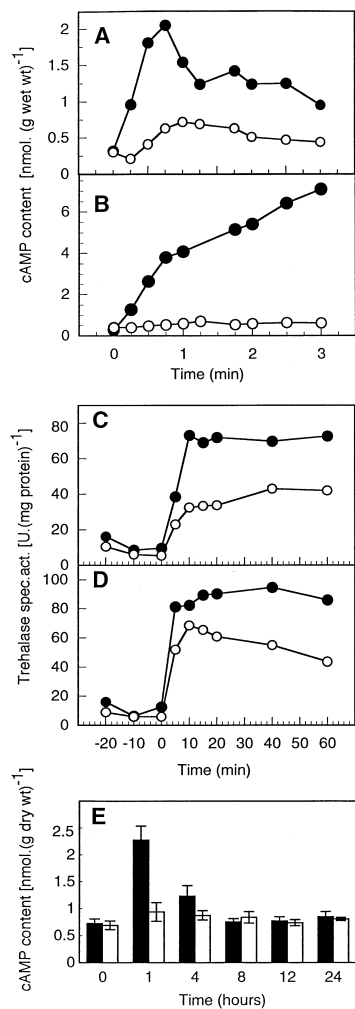
*Identification of the mutation as a A5627T substitution*

Sequencing of the 3'-terminal fragment revealed a substitution of adenine for thymine at position 5627. Sequencing of the other two fragments confirmed that there was no mutation present. The A5627T mutation was confirmed in all *lcr1* derivatives and the wild-type adenine nucleotide in all wild-type derivatives of the following tetrads: 7117A-D, 7103A-D and 7106A-D. DNA sequence analysis of the area around nucleotide 5627 in four of the chimeric constructs showed that the WWW construct contained an adenine at position 5627, whereas the MMM, WWM and WMM constructs contained a thymine at this position.

The A5627T mutation causes a substitution in adenylate cyclase of lysine at position 1876 for methionine. This amino acid is located near the end of the catalytic domain (Kataoka *et al.*, 1985) and could therefore have an important influence on the activity of the enzyme. Interestingly, however, the mutation does not affect  $Mn^{2+}$ -dependent adenylate cyclase activity but strongly reduces  $GTP/Mg^{2+}$ -dependent activity.

*Introduction of the A5627T mutation into a wild-type strain, cAMP responses in vivo and adenylate cyclase activity in vitro*

We have introduced the A5627T mutation in the *CYR1* gene of the wild-type strain W303-1A by replacement of the C-terminal third of the wild-type gene by the corresponding part of the *cyr1*<sup>met1876</sup> (*lcr1*) allele (see *Experimental procedures*). The presence of only the A5627T mutation in the *CYR1* gene of the W303-1A strain was confirmed by sequencing. The resulting strain GD1 lacked



**Fig. 3.** Intracellular cAMP level as a function of time after the addition of 100 mM glucose (A) or 2 mM 2,4-dinitrophenol (B). Wild-type strain W303-1A (●), isogenic *cyr1*<sup>met1876</sup> (*lcr1*) mutant strain GD1 (○). Trehalase activity as a function of time after the addition of 100 mM glucose (C) or 2 mM 2,4-dinitrophenol (D). Basal intracellular cAMP level during diauxic growth on glucose (E). The glucose was exhausted between 8 and 12 h. Black bars, wild-type strain W303-1A; open bars, isogenic *cyr1*<sup>met1876</sup> (*lcr1*) mutant strain GD1.

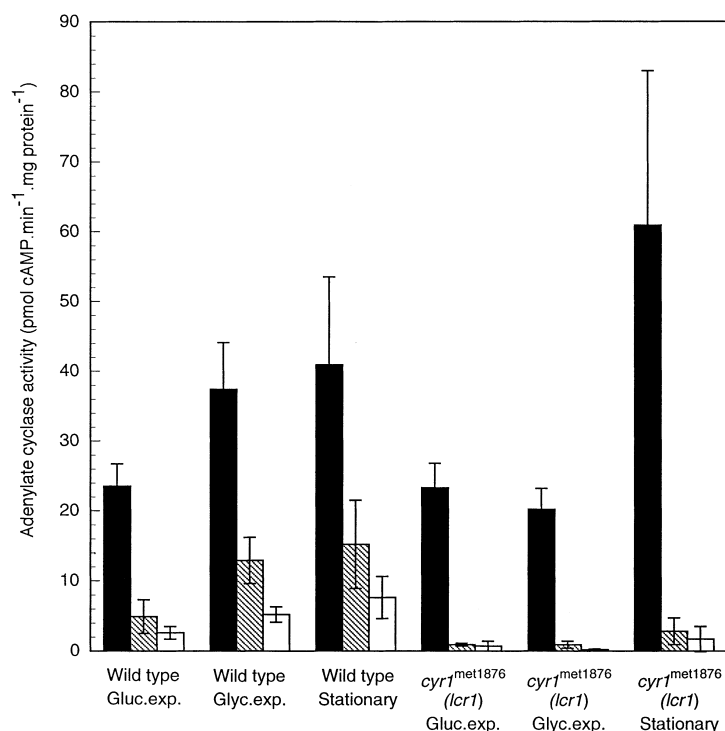
both the glucose- and acidification-induced cAMP responses, although a small increase was still observed with glucose (Fig. 3A and B). During the investigation of cAMP responses in the original and tetrad-derived *lcr1* strains, similar small increases in the cAMP level after the addition of glucose were occasionally observed in some strains (results not shown). These results indicated that the K1876M mutation in adenylate cyclase is able to largely eliminate the cAMP responses by itself. Given the previous results that linked this phenotype to the C-terminal third of adenylate cyclase, it shows that the K1876M mutation alone is responsible for the elimination of the cAMP responses in the *lcr1* mutant strains.

Determination of adenylate cyclase activity in isolated plasma membranes of the W303-1A wild-type strain and the isogenic *cyr1*<sup>met1876</sup> (*lcr1*) mutant strain GD1 showed that, in the latter strain, Mn<sup>2+</sup>-dependent activity was similar to that in the wild-type strain, but Gpp(NH)p/Mg<sup>2+</sup>- and Mg<sup>2+</sup>-dependent activity were strongly reduced (Fig. 4). This was true for the three different growth conditions used. This indicates that the absence of the agonist-induced cAMP responses is not caused by a general reduction in adenylate cyclase activity, but by a specific change in responsiveness of the adenylate cyclase to agonist stimulation. Gpp(NH)p/Mg<sup>2+</sup>- and Mg<sup>2+</sup>-dependent activity are a measure, respectively, of maximal and basal stimulation of adenylate cyclase by GTP-dependent G-proteins.

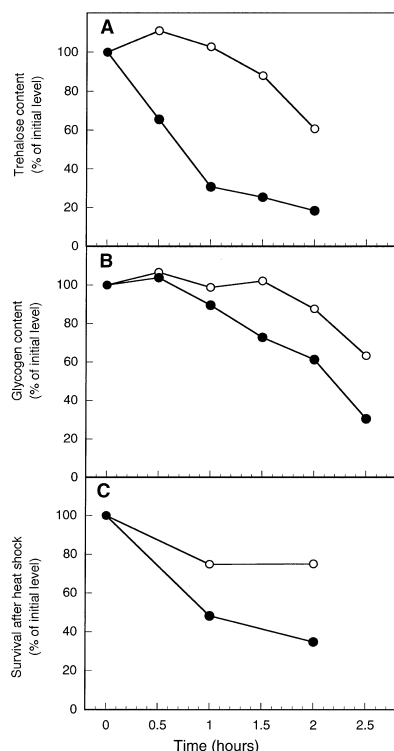
#### Phenotype of a strain lacking the glucose- and acidification-induced cAMP responses

Two isogenic strains, W303-1A and GD1, were now available that allowed us to investigate (without side-effects possibly caused by mixed genetic backgrounds, the presence of suppressor mutations or multicopy suppressor genes) the physiological relevance of agonist-induced cAMP signalling. Glucose- and intracellular acidification-triggered activation of trehalase are considered to be mediated by the cAMP increases caused by these agonists (Thevelein, 1991). In both cases, the extent of trehalase activation was reduced in the *cyr1*<sup>met1876</sup> (*lcr1*) mutant (Fig. 3C and D). It was not abolished, indicating the involvement of additional factors or the presence of undetected local cAMP increases. Recently, we have shown that the lag phase of growth in yeast is associated with a transient increase in the basal cAMP level. Also, this transient cAMP increase was abolished in the *cyr1*<sup>met1876</sup> (*lcr1*) mutant (Fig. 3E). This might be the reason for the slightly longer lag phase of the mutant. In addition to the rapid mobilization of trehalose, triggered by the activation of trehalase, mobilization of glycogen and rapid loss of stress resistance are also important characteristics of the adaptation from gluconeogenic/respirative growth to fermentative growth. These effects are also thought to be mediated by a cAMP-triggered protein phosphorylation cascade that occurs after the addition of glucose (Thevelein, 1988). We now show that, for each of these three characteristics, a significant delay occurs in the *cyr1*<sup>met1876</sup> (*lcr1*) mutant (Fig. 5). This is consistent with a role for glucose-induced cAMP signalling in the stimulation of the transition to fermentative growth.

Growth of the isogenic strains W303-1A and GD1 on different carbon sources showed a slightly, but reproducibly longer lag phase and higher final cell density on rapidly fermented sugars for the *cyr1*<sup>met1876</sup> (*lcr1*) mutant (glucose: Fig. 6A; fructose: results not shown) and a slightly shorter



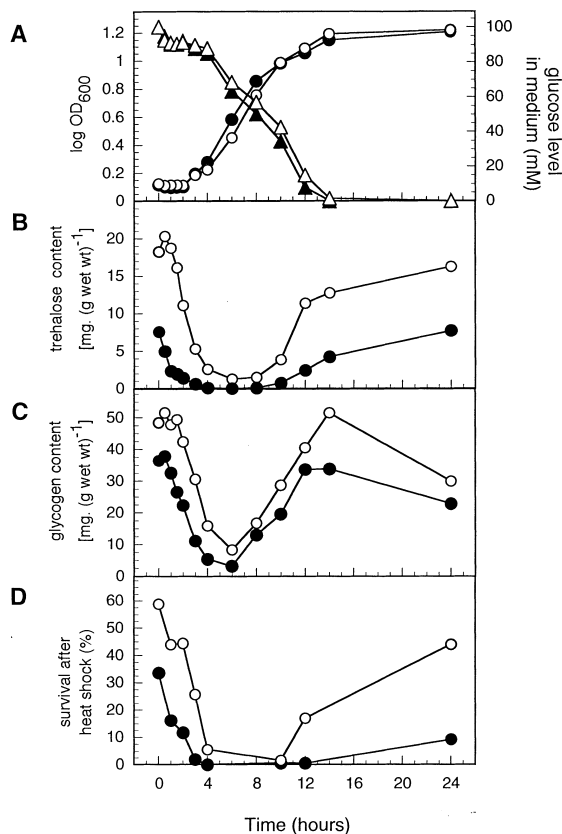
**Fig. 4.** Adenylate cyclase activity measured in isolated plasma membranes of the wild-type strain (W303-1A) and the isogenic *cyr1<sup>met1876</sup> (lcr1)* mutant strain (GD1). Membranes from cells growing exponentially on glucose or on glycerol and from stationary-phase cells (grown on glucose) were used. Black bars, activity in the presence of 2.5 mM MnCl<sub>2</sub>; striped bars, activity in the presence of 2.5 mM MgCl<sub>2</sub> + 100 μM Gpp(NH)p; open bars, activity in the presence of 2.5 mM MgCl<sub>2</sub>.



**Fig. 5.** Glucose-induced mobilization of trehalose (A) and glycogen (B) and glucose-induced loss of heat resistance (C) in glycerol-grown cells. Wild-type strain W303-1A (●), isogenic *cyr1<sup>met1876</sup> (lcr1)* mutant strain GD1 (○).

lag phase and lower final cell density on non-fermentable carbon sources (glycerol, ethanol: results not shown). On galactose, there was no difference between the two strains. These results would be consistent with a stimulatory role for glucose-induced cAMP signalling in the initiation of fermentative growth. Although the difference under our growth conditions was small (e.g. Fig. 6A), it cannot be excluded that, under certain conditions in the natural environment, this difference would be more pronounced and of selective advantage. The slightly longer lag phase of the *cyr1<sup>met1876</sup> (lcr1)* mutant correlated with a slightly slower consumption of glucose in the medium (Fig. 6A).

It is also well known that, during diauxic growth of yeast cells on glucose, a general fluctuation occurs in PKA-controlled properties. The trehalose and glycogen content and heat resistance drop during the initiation of fermentative growth to reach a minimum during mid-exponential phase, after which they increase again to reach a maximum during growth on ethanol and the subsequent stationary phase (for reviews, see Broach and Deschenes, 1990; Thevelein, 1992; 1994). Figure 6B–D shows that the initial and final levels of trehalose, glycogen and heat resistance were elevated in the *cyr1<sup>met1876</sup> (lcr1)* mutant, while the difference between the two strains was small at early/mid-exponential phase. More important, however, is the observation that, in the strain lacking glucose-induced cAMP signalling, the general fluctuation pattern in the three parameters during diauxic growth was not



**Fig. 6.** Growth and glucose level in the medium (A), trehalose (B) and glycogen (C) content and heat shock resistance (D) of the cells during diauxic growth. Wild-type strain W303-1A (filled symbols), isogenic *cyr1*<sup>met1876</sup> (*lcr1*) mutant strain GD1 (open symbols). OD<sub>600</sub>, trehalose content, glycogen content and heat shock resistance (●, ○), glucose level in the medium (▲, △).

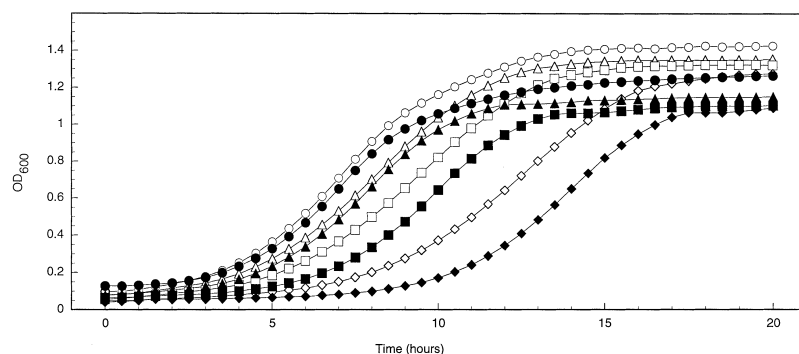
fundamentally affected. It was even more pronounced in the mutant than in the wild type. This indicates that glucose-induced cAMP signalling is not responsible for this general fluctuation in PKA-controlled properties.

The physiological function of the strong stimulating effect of intracellular acidification on the cAMP level is not clear. We found that growth inhibition by the weak acid preservative sorbic acid (results not shown) and by

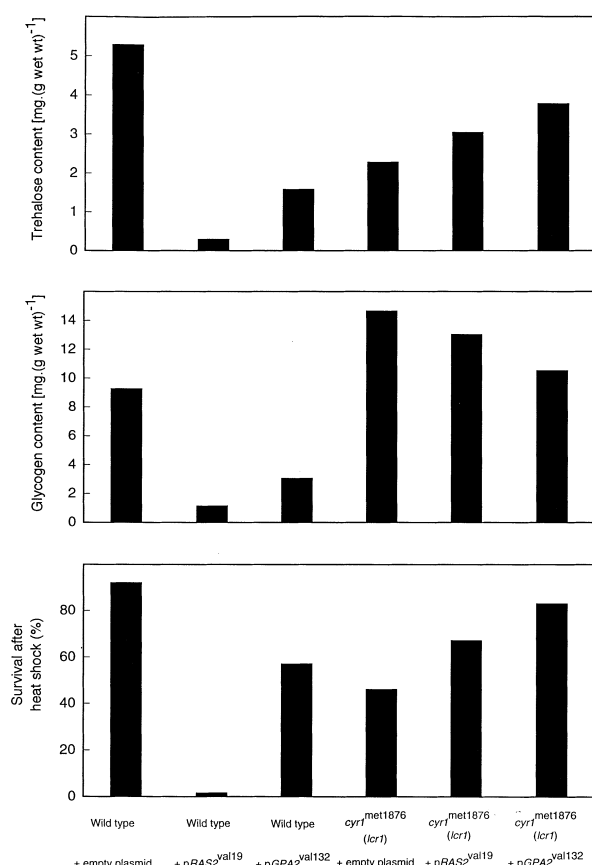
acetic acid (Fig. 7) is significantly reduced in the *cyr1*<sup>met1876</sup> (*lcr1*) mutant. The mutant showed better growth in the presence of these inhibitors than the wild-type strain. This suggests that at least part of the inhibition results from overstimulation of cAMP accumulation by the intracellular acidification effect. As recent evidence has suggested that sorbic acid might not act as a classic weak acid preservative (Stratford and Anslow, 1998), we also tested acetic acid. However, the results obtained with sorbic acid and acetic acid were very similar, except that sorbic acid was effective at about 60 times lower concentrations.

#### Suppression of *Ras2*<sup>val19</sup> and *Gpa2*<sup>val132</sup> by the *cyr1*<sup>met1876</sup> (*lcr1*) mutation

*Ras2*<sup>val19</sup> causes strong constitutive activation of the cAMP pathway (Toda *et al.*, 1985), and recent work has shown that the dominant alleles *Gpa2*<sup>ala273</sup> (Xue *et al.*, 1998) and *Gpa2*<sup>val132</sup> (Kraakman *et al.*, 1999) also cause constitutively low stress resistance, a well-known PKA-controlled property. We show that the *cyr1*<sup>met1876</sup> (*lcr1*) mutation partially suppresses the effect of both *Ras2*<sup>val19</sup> and *Gpa2*<sup>val132</sup> for trehalose content, glycogen content and heat resistance. This suppression was more pronounced in cells grown in minimal medium (Fig. 8) compared with cells grown in rich medium (results not shown). As opposed to the results for cells in rich medium (Fig. 6), when the cells were grown in minimal medium to stationary phase, the *cyr1*<sup>met1876</sup> (*lcr1*) mutant did not show a higher final trehalose content and heat resistance than the wild-type strain. Only the glycogen content was higher (Fig. 8). When the wild-type strain was transformed with the *RAS2*<sup>val19</sup>- or *GPA2*<sup>val132</sup>-containing plasmid, a strong drop in trehalose and glycogen content and in heat resistance was observed compared with the wild-type strain with the empty plasmid (Fig. 8). On the other hand, in the *cyr1*<sup>met1876</sup> (*lcr1*) mutant, the two alleles were unable to lower trehalose and glycogen content or heat resistance (Fig. 8). This indicates that the *cyr1*<sup>met1876</sup> (*lcr1*) allele effectively



**Fig. 7.** Growth on YPD in the presence of acetic acid. Wild-type strain W303-1A (filled symbols), isogenic *cyr1*<sup>met1876</sup> (*lcr1*) mutant strain GD1 (open symbols). Acetic acid concentrations: 0 mM (●, ○); 40 mM (▲, △); 60 mM (■, □); 80 mM (◆, ◇).



**Fig. 8.** Suppression by the *cyr1*<sup>met1876</sup> (*lcr1*) mutation of the effect of *Ras2*<sup>val19</sup> and *Gpa2*<sup>val132</sup> on PKA targets: trehalose (A) and glycogen (B) content and heat shock resistance (C). The cells were grown on minimal medium into stationary phase.

counteracts the overactivating effect of *RAS2*<sup>val19</sup> or *GPA2*<sup>val132</sup>. This result also supports the theory that *Gpa2* acts upstream of adenylate cyclase for cAMP signalling through the cAMP pathway. Although direct activation of adenylate cyclase by *Gpa2* is its most likely mechanism of action based on the analogy with G-protein control of mammalian adenylate cyclase, it has been difficult to establish this in yeast because of the lethality caused by adenylate cyclase deletion and the essential requirement of the Ras proteins for adenylate cyclase activity.

## Discussion

*The K1876M mutation in adenylate cyclase largely abolishes agonist-induced cAMP signalling*

Our results show that a K1876M mutation in adenylate cyclase largely abolishes the cAMP responses *in vivo* upon addition of glucose or intracellular acidification, without significantly reducing the basal cAMP level measured *in vivo*. Also, the Mn<sup>2+</sup>-dependent activity of adenylate

cyclase *in vitro* is not significantly affected, whereas GTP/Mg<sup>2+</sup>-dependent activity is strongly reduced. Hence, both *in vivo* and *in vitro* data indicate that the *cyr1*<sup>met1876</sup> (*lcr1*) mutation does not cause a general non-specific drop in the catalytic activity of adenylate cyclase, but rather that agonist stimulation of adenylate cyclase is specifically absent in the mutant enzyme. The most straightforward explanation of our observations is that the mutation specifically affects the regulation of the enzyme by the proteins responsible for agonist-induced activation. If this interpretation is correct, the data would tend to indicate that, in the case of glucose control and control by intracellular acidification, the proteins involved act on a similar site of adenylate cyclase or that the lysine at position 1876 plays a key role in the subsequent response of the adenylate cyclase catalytic domain to this interaction. This interpretation is supported by the finding that the effect of both *Ras2*<sup>val19</sup> and *Gpa2*<sup>val132</sup> can be counteracted by the K1876M mutation. An alternative explanation is that the K1876M mutation causes a conformation mimicking constitutive feedback inhibition of adenylate cyclase by PKA. The enzyme is known to be under strong feedback inhibition by PKA (Nikawa *et al.*, 1987). The magnitude and persistence of the glucose-induced cAMP signal correlate negatively with PKA activity (Mbonyi *et al.*, 1990), and high PKA activity abolishes both the glucose- and the acidification-induced cAMP responses (Ma *et al.*, 1999). There are two arguments, however, against this alternative hypothesis. The first is that strains with constitutively high PKA activity (caused by a deletion of the *BCY1*-encoded regulatory subunit) have a lower cAMP level than the basal level in the *cyr1*<sup>met1876</sup> (*lcr1*) strains. The second is that the *cyr1*<sup>met1876</sup> (*lcr1*) mutation does not cause a general reduction in catalytic activity of adenylate cyclase but a reduction specifically in Mg<sup>2+</sup>/GTP-dependent activity.

The catalytic domain of *S. cerevisiae* adenylate cyclase was defined as residing approximately between amino acids 1609 and 1890 based on the inability of truncated enzymes lacking residues more upstream of residue 1890 to complement a *cyr1* mutant (Yamawaki-Kataoka *et al.*, 1989). Previously, other mutations have been identified in or near the catalytic domain of adenylate cyclase. A mutation at position 1651 makes the catalytic activity independent of Ras proteins and, at the same time, caused a stronger stimulation by Ras (De Vendittis *et al.*, 1986). A mutation at position 1547 changes the specificity for Ras interaction (Marshall *et al.*, 1988). The catalytic domain of *S. cerevisiae* and *Schizosaccharomyces pombe* adenylate cyclase show about 60% homology, while the rest of the enzyme is less conserved (Yamawaki-Kataoka *et al.*, 1989). Interestingly, the lysine-1876 residue is conserved in *S. pombe* adenylate cyclase. It is located in the most downstream domain conserved

between *S. cerevisiae* and *S. pombe* adenylate cyclase, which ends around residue 1890 (Yamawaki-Kataoka *et al.*, 1989). Hence, this last conserved domain could play an important role in the regulation of adenylate cyclase.

#### *Physiological significance of agonist-induced cAMP signalling*

Up to now, it has been difficult to assess the physiological significance of glucose-induced cAMP signalling because of the lack of strains that specifically affected agonist-induced activation of cAMP synthesis rather than the basal capacity of cAMP synthesis. The results with the *cyr1<sup>met1876</sup> (lcr1)* strain GD1 show that the absence of glucose-induced cAMP signalling clearly affects the rapid changes in several PKA targets that are associated with the transition from gluconeogenic/respirative growth to fermentative growth. The activation of trehalase was reduced. Mobilization of trehalose and glycogen and loss of stress resistance were significantly delayed. These results support the conclusion that glucose-induced cAMP signalling plays a role in stimulating the adaptation to growth on glucose. They fit with the previous observations that deletion of either *Gpa2* or *Gpr1*, which are both required for glucose-induced cAMP signalling, also delays glucose-induced changes in PKA targets (Colombo *et al.*, 1998; Kraakman *et al.*, 1999).

The effect of the *cyr1<sup>met1876</sup> (lcr1)* mutation was only quantitative and largely limited to the transition phase to fermentative growth. During diauxic growth, there was only a little difference from the wild-type strain. This fits with the presence of the *cyr1<sup>met1876</sup> (lcr1)* mutation in laboratory strains considered to be wild-type strains. The absence or, at least, the strong reduction in glucose-induced cAMP signalling does not eliminate the establishment of the typical PKA-controlled phenotype in glucose-growing cells. Moreover, the typical fluctuation in these properties during diauxic growth was even accentuated in the *cyr1<sup>met1876</sup> (lcr1)* mutant. This indicates that glucose-induced cAMP signalling is not responsible for the difference in these properties between cells growing on glucose and cells deprived of glucose. This is in agreement with the observation that, in *gpa2Δ* and *gpr1Δ* mutants also, the general fluctuation in PKA-controlled properties during diauxic growth is still present (Colombo *et al.*, 1998; Kraakman *et al.*, 1999). This conclusion also fits with the previous proposal that another glucose-dependent pathway, called the fermentable growth medium-induced (or FGM) pathway, is responsible for establishing the striking differences in PKA-controlled properties (Thevelein, 1994).

The *cyr1<sup>met1876</sup> (lcr1)* strain GD1 also displayed a small but reproducible increase in the length of the lag phase, which might be related to the absence of the transient

increase in the basal cAMP level during the lag phase. This cAMP increase has only recently been linked specifically to the lag phase of growth, whereas it was previously considered to reflect the level of glucose in the medium (Ma *et al.*, 1997). It might play a role in stimulating exit from stationary phase. A possible role of the cAMP pathway in outgrowth from stationary phase has been suggested (Tatchell, 1993).

Up to now, the physiological significance of acidification-induced cAMP signalling has been rather obscure. Its role might be limited to carbon-starved cells that are known to consume storage carbohydrates (Lillie and Pringle, 1980) and in which acidification is known to trigger the mobilization of storage carbohydrates (Berke and Rothstein, 1957). In carbon-starved cells of the *cyr1<sup>met1876</sup> (lcr1)* mutant strains, 2,4-dinitrophenol-induced trehalose mobilization was retarded compared with the wild-type strain, in agreement with a role for the acidification-induced cAMP increase in the stimulation of trehalose mobilization. Under the conditions of diauxic growth and absence of carbon starvation, as performed in our experiments, the changes caused by the *cyr1<sup>met1876</sup> (lcr1)* mutation in the PKA-controlled properties probably resulted from the absence of glucose-induced rather than acidification-induced cAMP signalling.

Our results show that the *cyr1<sup>met1876</sup> (lcr1)* strain is more resistant to growth inhibition by the weak acid preservative sorbic acid and by acetic acid. This supports the idea that, *in vivo*, the *cyr1<sup>met1876</sup> (lcr1)* mutation prevents the toxic effect caused by intracellular acidification to some extent. This result indicates that stimulation of the cAMP pathway by intracellular acidification is part of the mechanism responsible for growth inhibition by sorbic acid in yeast. Although a recent report provided evidence that sorbic acid does not inhibit yeast as a classic weak acid preservative (Stratford and Anslow, 1998), the results that we obtained with sorbic acid and acetic acid were very similar.

#### *The *cyr1<sup>met1876</sup> (lcr1)* allele as a tool for studying the signalling function of the cAMP pathway*

The lethal phenotype of mutants in adenylate cyclase and other essential components of the cAMP pathway has been very convenient for epistasis studies on the position of the different components in the pathway. However, there is more and more doubt as to whether the essential requirement of the cAMP pathway reflects an essential requirement for cAMP signalling. Deletion of *Gpa2* or *Gpr1*, for instance, abolishes glucose-induced cAMP signalling but is not lethal (Colombo *et al.*, 1998; Kraakman *et al.*, 1999). Hence, epistasis studies based on the essential character of adenylate cyclase, e.g. using the temperature-sensitive *cdc35* mutants, could lead to erroneous

results with respect to the proper localization of non-essential components of the cAMP pathway that are solely involved in cAMP signalling. Our results show that the *cyr1<sup>met1876</sup>* (*lcr1*) allele provides a convenient means of abolishing, or at least greatly reducing, cAMP signalling without causing lethality. The constitutive signalling effects of both *Ras2<sup>val19</sup>* and *Gpa2<sup>val132</sup>* are partially suppressed by the *cyr1<sup>met1876</sup>* (*lcr1*) mutation, indicating that both of them act upstream of adenylate cyclase. At present, it is unclear what part of adenylate cyclase interacts with Gpa2. Our results are in agreement with at least partial overlap between the sites interacting with Ras and Gpa2, as the *cyr1<sup>met1876</sup>* (*lcr1*) mutation partially suppresses the effect of both constitutive alleles, although alternative interpretations cannot be excluded at this moment.

### Conclusions

The lysine-1876 residue in *S. cerevisiae* adenylate cyclase is essential for agonist-induced cAMP signalling *in vivo*. Mutation of this residue does not reduce maximal adenylate cyclase activity *in vitro*, but strongly reduces GTP-stimulated activity. This indicates that mutation of the residue specifically prevents agonist-induced cAMP signalling through an effect on G-protein activation of the enzyme

rather than basal activity. Elimination of glucose activation of cAMP synthesis *in vivo* by introduction of the *cyr1<sup>met1876</sup>* (*lcr1*) mutation in adenylate cyclase delays glucose-induced changes in PKA targets associated with the adaptation to growth on glucose. However, it does not eliminate, or even reduce, the typical variation in PKA-controlled phenotypic properties during diauxic growth, which must, therefore, be caused by a cAMP signalling-independent pathway.

### Experimental procedures

#### Strains and growth media

*S. cerevisiae* strains used in this study are shown in Table 1. Tetrads MV7103A–D and MV7106A–D were derived from a cross between SP1 and ENY.cat80-7A. Tetrad MV7117A–D was derived from a cross between SP1 and MV7103B. The presence of the *can1* marker was not checked in these tetrads. MV7143 is a diploid strain obtained by crossing MV7117A and Be333. Strains MV7159 and MV7161 were obtained by transformation of MV7117C and MV7117D, respectively, with plasmid YCplac33YACE1. Strain GD1 was constructed by insertion of the vector Ylplac211(*URA3*) containing an *Xba*I–*Bam*HI fragment of the *cyr1<sup>met1876</sup>* (*lcr1*) mutant allele and cut with *Cl*aI into strain W303-1A. Subsequently, the strain was plated on a 5-fluoro-orotic acid plate, and spontaneous *ura<sup>-</sup>* revertants were screened for the presence of the A562T mutation in the *CYR1* gene.

**Table 1.** *Saccharomyces cerevisiae* strains used in this work.

Strain	Relevant genotype	Complete genotype	Source and/or reference
ENY.cat80-7A	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> leu2-3112 his3-<math>\delta</math>1 ura3-52 CAT80 MAL2-8<sup>c</sup> MAL3 SUC3</i>	K.-D. Entian (Frankfurt, Germany)
ENY.cat80-8A	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> leu2-3112 ura3-52 CAT80 MAL2-8<sup>c</sup> MAL3 SUC3</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	K.-D. Entian (Frankfurt, Germany)
SP1	Wild type	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3 ade8 can1</i>	M. Wigler (Cold Spring Harbor, NY, USA)
CEN.PK2-1C	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3 MAL2-8<sup>c</sup> SUC2</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	E. Boles (Düsseldorf, Germany)
MV7103A	<i>CYR1</i>	<i>MAT<math>\alpha</math> his3 leu2 ura3 ade8</i>	This work
MV7103B	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> leu2 trp1 ura3</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work
MV7103C	<i>CYR1</i>	<i>MAT<math>\alpha</math> leu2 ura3 ade8</i>	This work
MV7103D	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work
MV7106A	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work
MV7106B	<i>CYR1</i>	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3</i>	This work
MV7106C	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> leu2 trp1 ura3 ade8</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work
MV7106D	<i>CYR1</i>	<i>MAT<math>\alpha</math> leu2 trp1 ura3 ade8</i>	This work
MV7117A	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> leu2 trp1 ura3 ade8</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work
MV7117B	<i>CYR1</i>	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3</i>	This work
MV7117C	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	<i>MAT<math>\alpha</math> leu2 trp1 ura3</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work
MV7117D	<i>CYR1</i>	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3 ade8</i>	This work
MV7143	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )/ <i>cdc35-10</i>	<i>MAT<math>\alpha</math>/MAT<math>\alpha</math> leu2 LEU-2 trp1/ TRP1 ura3/URA3 ade8/ADE8</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )/ <i>cdc35-10</i>	This work
MV7159	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> ) + YCpCYR1	<i>MAT<math>\alpha</math> leu2 trp1 ura3</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> ) + YCplac33YACE1 ( <i>CYR1</i> )	This work
MV7161	<i>CYR1</i> + YCpCYR1	<i>MAT<math>\alpha</math> his3 leu2 trp1 ura3 ade8</i> + YCplac33YACE1 ( <i>CYR1</i> )	This work
Be333	<i>cdc35-10</i>	<i>MAT<math>\alpha</math> cdc35-10</i>	F. Hilger (Gembloux, Belgium)
W303-1A	Wild type	<i>MAT<math>\alpha</math> leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal</i>	Thomas and Rothstein (1989)
GD1	<i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> ) (isogenic to W303-1A)	<i>MAT<math>\alpha</math> leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal</i> <i>cyr1<sup>met1876</sup></i> ( <i>lcr1</i> )	This work

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.

Growth media were composed of 2% bacto peptone (2%), yeast extract (1%) and 2% glucose (YPD) or 3% glycerol (YPGlycerol). Minimal media (SD) were as specified by Sherman *et al.* (1986). They were used to determine auxotrophic markers and to maintain plasmids in transformed strains.

### Plasmids

Plasmid pUC19\* was constructed from pUC19 (Yanisch-Perron *et al.*, 1985) by deletion of the *SacI*–*SmaI* fragment of the multicloning site. Plasmid pYACE1 (Feger *et al.*, 1991) was kindly provided by O. Fasano (Palermo) and contains the *CYR1* gene on an 8.47 kb *Sau3A*–*Sau3A* fragment in plasmid YEp26 (Broach *et al.*, 1979). Plasmids YCplac33, Ylplac128, Ylplac204 and Ylplac211 have been described by Gietz and Sugino (1988). Plasmid YCplac33YACE1 was constructed by insertion of the *CYR1* gene on an 8.5 kb *SphI*–*BamHI* fragment derived from pYACE1 into YCplac33.

The YCplac33 plasmids containing different chimeric constructs of the wild-type *CYR1* and *lcr1* mutant allele were made by replacement of the *SphI*–*Asp718*, *Asp718*–*NcoI* or *NcoI*–*BamHI* fragment in the YCplac33YACE1 plasmid ('pWWW') by the corresponding fragments obtained from parts of the *lcr1* mutant allele cloned in the vector Ylplac211. These parts were a 5.2 kb *SphI*–*PstI*, a 4.9 kb *PstI*–*NcoI* and a 1.4 kb *NcoI*–*SnaBI* fragment. They were recovered separately by plasmid eviction. The chimeric constructs were first made in the pUC19\* vector, cut out with *SphI* and *BamHI* and cloned into the *SphI*–*BamHI* sites of the multicloning site of vector YCplac33.

For insertion of a plasmid of the Ylplac series at different positions in the *CYR1* promoter of the wild-type strain SP1, the following plasmids were constructed and restriction sites used: Ylplac211 + 2.2 kb *HindIII*–*EcoRI* fragment of *CYR1*, *HindIII* (–1790); Ylplac204 + 2 kb *HincII*–*EcoRI* fragment of *CYR1*, *HincII* (–1590); Ylplac128 + 1.25 kb *AccI*–*EcoRI* fragment of *CYR1*, *AccI* (–840); and Ylplac211 + 0.93 kb *DraI*–*EcoRI* fragment of *CYR1*, *DraI* (–526).

The *RAS2*<sup>val19</sup> allele (Toda *et al.*, 1985) and the *GPA2*<sup>val132</sup> allele (Kraakman *et al.*, 1999) were introduced into the wild-type strain W303-1A and the isogenic *cyr1*<sup>met1876</sup> (*lcr1*) mutant (GD1) on a YCplac33 plasmid (Gietz and Sugino, 1988). The same strains transformed with the empty plasmid were used as controls.

### Recombinant DNA methods and genetic analysis

Standard methods were used for transformation, DNA extraction, restriction analysis, Southern blotting and polymerase chain reaction (PCR) (Sambrook *et al.*, 1989). DNA sequence analysis was performed according to Sanger *et al.* (1977), using the T7 sequencing kit from Pharmacia. Tetrad analysis was performed according to standard procedures (Sherman *et al.*, 1986). Cloning of the unknown insert in the *CYR1* promoter of the *lcr1* mutant was performed using plasmid eviction.

For this purpose, the 2.6 kb *SphI*–*EcoRI* fragment of the *CYR1* promoter and gene was introduced into the corresponding site in the Ylplac211 vector and integrated into an *lcr1* strain (MV7117C) after linearization with *PstI*. Genomic DNA of a positive transformant was cut with *SphI*, and Southern blot analysis revealed a fragment of 9.5 kb. The DNA fragment was ligated and transformed into *Escherichia coli*. After recovery of the plasmids, a restriction map was made of the insert and used to obtain subclones that were inserted into pUC19.

### Growth measurements and determination of heat shock resistance

For comparison of the growth rate of W303-1A and GD1, stationary-phase cultures were diluted 100-fold into YP medium with different carbon sources as specified, and the OD<sub>600</sub> was recorded with a turbidometric analyser incubated at 30°C (Bioscreen; Labsystems). Two duplicates of three independent cultures were measured simultaneously. Growth in the presence of the weak acid preservative, sorbic acid, or in the presence of acetic acid was measured in the same way in YPD medium. In this case, all growth curves were determined with four replicate cultures. For determination of heat shock resistance, 100 µl samples were taken from the culture at the indicated time points and heated for 20 min at 51°C (or 15 min at 49°C in Fig. 7C). After cooling, aliquots were spread on nutrient plates, and colonies were counted after 3 days of growth at 30°C.

### Biochemical determinations

For determination of cAMP content, cells were grown on YPGlycerol until exponential phase, harvested and resuspended in 25 mM Mes buffer (pH 6). They were preincubated for 10 min before the addition of 100 mM glucose or 2 mM 2,4-dinitrophenol. Extraction and determination of cAMP were performed as described previously (Thevelein *et al.*, 1987b).

For trehalose and glycogen determination, cells were collected by filtration, washed once with cold water, weighed and frozen in liquid nitrogen. The pellets were resuspended in 0.5 ml of 0.25 M Na<sub>2</sub>CO<sub>3</sub> per 50 mg of cells and boiled at 95°C for 20 min. Samples were taken for trehalose determination, and the remainder was boiled for another 60 min. The samples for trehalose determination were spun down, and 10 µl of the clear supernatant was used. The samples for glycogen determination were mixed well and used directly (10 µl). All samples were neutralized by the addition of 5 µl of 1 N acetic acid. For trehalose determination, 5 µl of buffer (300 mM sodium acetate, 30 mM CaCl<sub>2</sub>, pH 5.4) and 20 µl of *Humicola* trehalase (360 U ml<sup>–1</sup>) (Neves *et al.*, 1994) were added and incubated for 45 min at 40°C. For glycogen determination, 5 µl of buffer (400 mM sodium acetate, pH 4.7) and 20 µl of amyloglucosidase (0.25 U) from *Aspergillus niger* (Boehringer Mannheim) were added and incubated for 2 h at 37°C. For all samples, the glucose liberated was measured in 30 µl of cleared supernatant using the glucose oxidase/peroxidase method.

Adenylate cyclase activity was assayed in purified yeast plasma membranes. Preparation of crude membranes was performed essentially as described by Mintzer and Field

(1995) with minor modifications. Adenylate cyclase was assayed in a reaction mixture containing 20 mM Mes, pH 6.2, 0.1 mM  $\text{MgCl}_2$ , 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 0.25 mM IBMX, 0.1 mg ml<sup>-1</sup> BSA, 0.25 mM cAMP, 1 mM [ $\alpha$ -<sup>32</sup>P]-ATP (final specific activity 100–150 c.p.m. pmol<sup>-1</sup>), 20 mM phosphocreatine, 0.25 mg ml<sup>-1</sup> creatine phosphokinase, 0.1 mM dithiothreitol (DTT), in the presence of 2.5 mM  $\text{MnCl}_2$  or 2.5 mM  $\text{MgCl}_2$  or 2.5 mM  $\text{MgCl}_2$  and 100  $\mu\text{M}$  Gpp(NH)p. The reaction was initiated by addition of the reaction mixture to the membranes (20–60  $\mu\text{g}$  of protein) in a final volume of 100  $\mu\text{l}$ . The tubes were immediately transferred to a 30°C water bath and incubated for 20 min. The reaction was stopped by the 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% SDS. Cyclic [<sup>3</sup>H]-AMP ( $\approx$  20 000 c.p.m.) was added to monitor sample recovery. [<sup>32</sup>P]-cAMP produced was determined as described by Salomon *et al.* (1974) with minor modifications.

Trehalase activity was determined in crude cell extracts as described previously (Pernambuco *et al.*, 1996). Glucose consumption in the medium was measured by the glucose oxidase/peroxidase method.

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