New roles for \textit{CDC25} in growth control, galactose regulation and cellular differentiation in \textit{Saccharomyces cerevisiae} \\
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Living organisms display large differences in stress resistance throughout their life cycles. To study the coordinated regulation of development and stress responses in exponentially growing yeast, mutants that displayed elevated heat-shock resistance at this stage were screened for. Here, two new mutant alleles of \textit{CDC25} in \textit{Saccharomyces cerevisiae}, \textit{cdc25-21} and \textit{cdc25-22}, are described. During exponential growth in glucose at 25 °C, these mutants are resistant to heat, oxidative, osmotic and ionic shock, accumulate stress-protein transcripts, show slow growth rates, thick cell walls and glycogen hyperaccumulation and lack cAMP signalling in response to glucose. Genetic and cellular analyses revealed that the stationary-phase phenotypes of \textit{cdc25-21} and \textit{cdc25-22} mutants are not due to entrance to a G0 state during exponential growth, but are the result of a prolonged G1 phase. It was found that, in the W303 background, \textit{CDC25} is dispensable for growth in glucose media. However, \textit{CDC25} is essential for growth in galactose, in non-fermentable carbon sources and under continuous incubation at 38 °C. In conclusion, the function of the catalytic, C-terminal domain of Cdc25p is not only important for fermentative growth, but also for growth in non-fermentable carbon sources and to trigger galactose derepression. \\

INTRODUCTION \\
When grown in liquid media that contain glucose as the carbon source, yeast populations of \textit{Saccharomyces cerevisiae} experience five well-defined stages in their growth curve: lag phase, exponential phase, diauxic shift, post-diauxic phase and stationary phase (Werner-Washburne et al., 1993). Along these phases, cells undergo distinct metabolic and developmental changes to cope with variations in the medium. The lag phase is a period in which cells adjust their metabolism to the new medium and prepare for cell proliferation (Tortora et al., 1986). During the exponential phase, cell division attains its fastest rate and growth is sustained mainly by fermentation. Cells at this stage are very sensitive to heat shock and other stresses. As glucose levels diminish and ethanol accumulates, cells enter the diauxic phase, their growth rate decreases and their heat-shock resistance increases. Once nutrients are exhausted, cells enter the stationary phase, where they reach the maximum level of thermotolerance and cease to proliferate (reviewed by Werner-Washburne et al., 1993; Thevelein & de Winde, 1999). \\

A large number of studies have implicated the cAMP/protein kinase A (PKA) pathway in the control of these physiological processes (reviewed by Thevelein & de Winde, 1999; Thevelein et al., 2000). During the exponential phase, the cAMP/PKA pathway downregulates glycogen and trehalose content, stress tolerance, cell-wall resistance to lyticase digestion and expression of genes that are controlled by \textit{STRE}-boxes in their promoters (Plesset et al., 1987; Van Dijck et al., 1995). The cAMP/PKA pathway is also involved in the control of cell-cycle progression at G1 or G0 (Drebot et al., 1990; Thevelein, 1992), although it is not clear how this is achieved. \\

cAMP levels control PKA activity by interacting with its regulatory subunit (Bcy1p), which dissociates from the catalytic subunits (Tpk1p, Tpk2p and Tpk3p), resulting in their activation (Toda et al., 1987a, b). cAMP levels are controlled by synthesis (through adenylate cyclase) and by degradation (through Pde1p and Pde2p phosphodiesterases) (Ma et al., 1999). Adenylate cyclase is regulated by the monomeric G-proteins Ras1p and Ras2p, as well as by Srv2p (Fedor-Chaiken et al., 1990). Activation of Ras proteins requires Cdc25p, a guanine nucleotide-exchange factor that interchanges GDP for GTP (Broek et al., 1987;
Lai et al., 1993). The Ira1p and Ira2p proteins downregulate Ras by enhancing its intrinsic GTPase activity. Cells containing mutations that cause a decrease in the activity of the cAMP/PKA pathway, such as in adenylate cyclase (cyr1) or Ras (ras2), show slow growth rates, accumulate storage carbohydrates and are heat shock resistant (Iida & Yahara, 1984; Tatchell, 1986; Iida, 1988). In contrast, mutants with an overactive PKA (e.g. bcy1, RAS2alv9) fail to arrest in G1 upon nutrient limitation, quickly lose viability in the stationary phase and are very sensitive to different stresses (reviewed by Thevelein & de Winde, 1999).

A cAMP-independent pathway for controlling PKA targets, referred to as the fermentable growth medium (FGM) pathway, has been proposed. The FGM pathway involves the Sch9p kinase, although its relation to PKA is still not well understood (Thevelein et al., 2000).

In this work, we describe two novel alleles of CDC25 (cdc25-21 and cdc25-22) that show conspicuous phenotypes at 25 °C: elevated tolerance to heat shock and other stresses, a slow growth rate, thick cell walls, glycogen hyperaccumulation, constitutive expression of stress genes and lack of rapid cAMP accumulation upon addition of glucose. These mutants are unable to grow in galactose, in glucose at 40 °C or in non-fermentable media at optimal temperatures. The cdc25-21 mutant lacks 612 aa from the C-terminus, where the catalytic domain resides, but remains viable in glucose. Furthermore, a W303 derivative (Φcdc25), in which the CDC25 gene has been deleted, is still viable in glucose. We demonstrate that these mutations do not drive cells into the G0 or stationary phase of the cell cycle, but rather that they spend a longer time in G1. Our results indicate that the catalytic C-terminus of Cdc25p plays important roles during fermentative growth in glucose, during growth in non-fermentable carbon sources and in derepression from a glucose-repressed state.

METHODS

Strains and plasmids. All yeast strains used in this work are described in Table 1. ScIBT2 and ScIBT3 strains were obtained after ethyl methanesulfonate (EMS) mutagenesis of strain W303a-LEU+ (see below). Derivatives of ScIBT2 and ScIBT3 were obtained after repeated backcrosses to W303-6b, gcl1 cdc25-21 and gcl1 cdc25-22 double mutants were obtained by tetrad dissection of crosses between strains GWK-9a and JF099 (cdc25-21) or JF410 (cdc25-22). pRY002 was constructed by digesting pFF08 (a plasmid isolated during the complementation of mutant cdc25-21 that contains part of the IMH1 locus plus the full CDC25, YLR311C and YLR312C loci) with PvuI and the 5-95 kb fragment was cloned in vector pBS KS+. A Sall digestion was then used to excise a 5-52 kb fragment that contained the CDC25 gene, 37 bp of YLR311C and 40 bp of IMH1. The protruding ends of this fragment were filled in by using the Klengow fragment and cloned into the EcoRV site of the centromeric vector pRS413 (Sikorski & Hieter, 1989). Plasmid pLA41 was a kind gift of Enzo Martegani (Rudoni et al., 2000).

Media and growth conditions. Yeast cells were grown at 25 °C unless otherwise indicated. SD medium with the appropriate supplements (Guthrie & Fink, 1991) was used in most assays and to maintain plasmids or confirm auxotrophies. YPDA medium was used to propagate strains without plasmids. SAC [0-67 % YNB (Difco) and 1 % potassium acetate plus the appropriate supplements] was used to test growth in acetate as the carbon source. SRAf [0-67 % YNB (Difco) and 2 % raffinose plus the appropriate supplements] was used to grow cells prior to galactose induction. Growth in glycerol as the carbon source was tested in YPGly [2 % bactopeptone, 1 % yeast extract, 3 % (v/v) glycerol and 2 % agar]. Growth in galactose as the carbon source was tested in SGal (0-67 % YNB and 2 % galactose plus the appropriate supplements). 5-Fluoroorotic acid selection was performed according to Boeke et al. (1984). The slow-growth phenotype of cdc25 mutants was always analysed carefully before starting a new inoculum. To start a new culture, only isolated and small colonies from a freshly grown Petri dish were used. Under these circumstances, revertants never constituted >0.1 % of the total population when grown to mid-exponential phase (an OD600 of 0-5). If revertants for normal growth arise and are not looked after, they become the predominant population after two or three subcultures.

Thermotolerance assays. To evaluate the basal or intrinsic level of thermotolerance, we used the method described by Nicolet & Craig (1991). Heat shocks were given by incubating the cells in a 50 °C water bath for 20 min at 250 r.p.m. To measure cell viability, plates were incubated for 3-4 days at 25 °C for colony counting. Colony counting was performed by using COVASIAM (Corkidi & Craig, 1991). Thermotolerance levels are expressed as the percentage of the number of colonies after a heat shock divided by the number of colonies in the untreated sample. Duplication times were calculated by using the exponential curve-fitting equation (Draper & Smith, 1981). Growth curves with correlation coefficients >0.99 were used for the calculation.

Other stress assays. Strains were grown at 25 °C in liquid YPDA medium to mid-exponential phase (an OD600 of 0-4) and treated as follows: for ionic stress treatment, LiCl was added from a sterile stock solution to a final concentration of 0-4 M and the culture was incubated for 24 h. Oxidative stress was tested by adding H2O2 to a final concentration of 20 mM and cultures were incubated for 1 h. Sorbitol was added from a sterile stock solution to a final concentration of 3-5 M and cultures were incubated for 2 h. In all cases, incubations during stress treatment were performed at 25 °C and 250 r.p.m. After stress treatment, cultures were diluted to an OD600 of 0-2 and serial dilutions up to 10−3 were made. To assess survival, 3 μl of each dilution was dropped onto YPDA plates and incubated at 25 °C for 2 days (for the wild-type strain) or 3 days (for mutant strains).

Mutagenesis of the wild-type yeast strain. Wild-type strain W303a-LEU+ was mutagenized with EMS according to the method of Lawrence (1991). This strain shows a survival rate of 0-01 % after a heat shock of 20 min at 50 °C. Given the relatively high number of false-positive, heat-shock-resistant cells in a wild-type culture, several rounds of selection were applied to the mutagenized population to enrich for true heat-shock-resistant mutants. Immediately after mutagenesis, cells were resuspended in YPDA medium and divided into 40 samples (1 ml each). Each sample was incubated for 24 h at 25 °C for recovery. Glycerol was added to a final concentration of 15 % (v/v) and samples were frozen at −70 °C until further use. For screening, 0-4 ml aliquots of some samples were used to inoculate 5 ml YPDA medium, grown to mid-exponential phase (an OD600 of 0-4-0-6) at 25 °C and heat-shocked at 50 °C for 20 min. To start a new round of selection, 0-5 ml of each heat-shocked culture was used to inoculate a new 5 ml culture in YPDA medium and the cycle was repeated successively four more times. After each round of heat shock, aliquots were taken to assess cell viability of the culture. After five heat-shock rounds, the wild-type population displayed a survival rate of 0-1 %, whereas the survival rate in
different aliquots of the mutagenized population ranged from 0-41 to 4-1%. Colonies arising from each aliquot were taken to ensure the isolation of mutants resulting from independent events.

**Genetic techniques and nucleic acid manipulations.** Genetic techniques and DNA manipulations were performed according to Guthrie & Fink (1991) and Sambrook et al. (1989), respectively. DNA sequencing was performed by using an automated sequencer (model ABI Prism 377-18; Applied Biosystems) with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit. For Northern blot assays, total RNA was prepared from exponentially grown liquid cultures in SD medium at 25°C or after a heat shock for 15 min at 39°C by the method of Collart & Oliviero (1993). Aliquots (15 μg) of total RNA were separated by electrophoresis on 1-2% agarose gels that contained formaldehyde, transferred to Hybond-N+ membranes (Amersham Biosciences) and hybridized as described by the manufacturer. HSP104, TPS1, CIT1, GDP1 and IPP1 transcripts were detected by using 32P-radiolabelled DNA probes.

<table>
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<th>Strain</th>
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*Obtained from the collection of the Yeast Deletion Project.
†Provided by Dr Gerry Johnston.
‡Provided by Dr Kelly Tatchell.
§Provided by Dr Susan Lindquist.
∥Provided by Dr Roberto Gaxiola. All other strains were obtained in this work.
Allele rescue and sequencing of cdc25-21 and cdc25-22 alleles. A derivative of pJF08 (see below) was used to rescue the mutant alleles in cdc25-21 and cdc25-22 mutants according to the method of Rothstein (1991). pJF08 is in plasmid YCpl50 and contains a 9-609 kbp insert of wild-type yeast genomic DNA that includes the whole CDC25 locus (see ‘ScIBT2, ScIBT3 and derivative strains are allelic to CDC25′ section in Results). PvuII digestion of pJF08 yields a 9-898 kbp fragment (pJF08–PvuII) that consists of the whole vector plus 1090 bp of the 5′ end and 858 bp of the 3′ end of the insert. In pJF08–PvuII, the entire CDC25 locus and its flanking regions are deleted. Both mutants were transformed with linear pJF08–PvuII DNA. Plasmids from the ura3- transformants were recovered for DNA sequencing.

Yeast transformation. Yeast transformations were performed according to the method of Gietz & Woods (2002). For cdc25 mutant strains, 50 ml cultures of YPDA medium were inoculated with small colonies isolated from fresh plates. When they reached mid-exponential phase (an OD600 of 0.4–0.6), cultures were heat-shocked at 50°C for 20 min to counterselect thermosensitive revertants that form large colonies. After heat shock, cultures were cooled to 25°C and 10 μg DNA of a wild-type genomic library in Ycp50 (Rose et al., 1987) was used to transform strain JF099. Cells were plated out in SD medium without uracil and screened after 4–5 days growth at 25°C. Wild-type colony size was selected. The wild-type genomic library was a kind gift of Dr Alicia González.

Construction of yeast strains with a full deletion of the CDC25 ORF. The integrative disruption cassette loxP–kanMX–loxP was used to generate strains in which the CDC25 gene was deleted (Guldener et al., 1996). The disruption cassette served as template in a PCR that included, as forward oligonucleotide, a hybrid containing a 45 bp sequence that is complementary to the region at positions 96–51 downstream of the TAA stop codon of the CDC25 ORF, followed by 22 bp of the complementary sequence at the 3′ end of the cassette. As reverse primer, a hybrid containing a 45 bp sequence that is complementary to the region at positions 96–51 downstream of the TAA stop codon of the CDC25 ORF, followed by 22 bp of the complementary sequence at the 3′ end of the cassette, was used. The PCR product was transformed into the wild-type diploid strains RV008, BY4743 and LRA. Transformants were selected in YPDA medium plus 0.5 μg/ml geneticin ml−1 (G418) and replica-plated on the same medium to counterselect false-positives. For growth in liquid media, 2 μg/ml geneticin ml−1 was used. Correct insertion of the cassette in the W303 tetrads was verified by PCR and sequence analysis (data not shown). CDC25-deleted mutants are referred to as Δcdc25.

cAMP determination. Cells were grown in SD medium with the appropriate supplements for 48 h. Cultures were centrifuged, resuspended in 0.67% YNB plus supplements (without glucose) and incubated for 4 h to achieve carbon deprivation. Cellular extracts were obtained by breaking the cells in 8% TCA. After several ether extractions, extracts were lyophilized and resuspended in 2 ml 0.1% Tris/HCl, 4 mM EDTA (pH 7.5). When necessary, the pH was adjusted to 7.5. Finally, 50 μl aliquots were taken for cAMP determination with the use of a [α-32P]cAMP assay system (Amersham Biosciences), according to the manufacturer’s instructions.

Electron microscopy. Cells were grown to mid-exponential phase (an OD600 of 0.4) and fixed at 4°C for 24 h in a buffer of 6% glutaraldehyde and 4% paraformaldehyde [in 40 mM K2HPO4/KH2PO4 (pH 6–5), 0.5 mM MgCl2]. Cells were post-fixed with 2% potassium permanganate for 2 h to highlight cell-wall and membrane structures. Spurr resin was used to include the samples. Samples were cut by using a Leica Ultracut R ultramicrotome. Observations were performed with a Zeiss EM900 transmission electron microscope at 80 kV.

Flow-cytometric analysis of DNA content. Cultures (4 ml) were grown to mid-exponential phase (an OD600 of 0.4) in SD medium with the appropriate supplements. Aliquots of 0.5 ml were fixed in 1:166 ml 100% ethanol (70% final concentration). Samples were stored at 4°C until further use. Cells were washed three times with 0.5 M sodium citrate, pH 7.0; 25 μl RNase (10 mg ml−1) was added and samples were incubated for 1 h at 37°C. Cells were centrifuged for 2 min at 6000 r.p.m. in an Eppendorf microfuge and resuspended in staining buffer [0.05 M sodium citrate (pH 7), 0.01 M NaCl, 0.5% Nonidet-P40 and 16 μg propidium iodide ml−1]. Samples were incubated for at least 6 h at 4°C. Finally, cells were pelleted as above, resuspended in 1.5 ml EDTA (10 mM, pH 8), sonicated for 15 s and analysed by flow cytometry using a Becton Dickinson flow cytometer. Data acquisition and analysis were done by using CellQuest software.

Lyticase cell-wall digestion assays. We modified the cell-wall sensitivity assay described by Martegani et al. (1984) using lyticase instead of glusulase. Lyticase (70 U ml−1; Sigma) was added to cells that had been resuspended in 10 mM Tris/HCl, 1 mM DTT to an OD600 of 0.7–0.8 and the OD600 was measured every 10 min.

Glycogen determination. Cultures grown on YPDA plates for 3 days at 25°C were stained with 5 ml of a solution that contained 0.2% iodine and 0.4% potassium iodide. Colour pictures were taken 1 min after addition of the iodine/iodide solution to assess the degree of staining (Toda et al., 1985).

Glucose, ethanol and oxygen determination. To estimate glucose and ethanol levels, cells were cultured in a 1 l reactor in YPDA medium at 25°C and 250 r.p.m. Sterile air was pumped in at a rate of 10 l min−1. The culture was inoculated at an OD600 of 0.05 and samples (5 ml) were taken at the indicated times. Cells were removed by centrifugation and the supernatant was used to quantify glucose and ethanol with a Yellow Spring Instruments biochemistry analyser (model 2700). Membranes YSI2365 and YSI2786 were used for glucose and ethanol determination, respectively. To measure oxygen consumption, cells were grown in batch cultures in YPDA medium at 25°C and 250 r.p.m. When cells reached the mid-exponential phase (an OD600 of 0.4), an aliquot was taken and placed in a sealed chamber with oxygen polarographic probe (Mettler Toledo) to monitor dissolved oxygen tension (DOT). Controls were done by injecting a stock solution of potassium cyanide to a final concentration of 1 mM immediately after sealing the chamber. The signal of the DOT probe was amplified and acquired by a Macintosh LC computer via a MacADIOS 411 interface (GW Instruments) and a data-acquisition program written in QuickBasic (Microsoft) (Palomares & Ramirez, 1996).

β-Galactosidase assay. Cells were transformed with plasmid pLA41 to express the lacZ reporter under the GAL1 promoter. Cells were streaked out on plates of SD or 5% his to avoid the accumulation of revertants in liquid cultures (only cells that formed small colonies were analysed from the Δcdc25 strain). Plates were incubated at 30°C for 3 days in SD or 5 days on 5% his. To induce the expression of β-galactosidase, collected cells were resuspended in 4 ml Sgal and incubated at 25°C for 75 min. Cultures were centrifuged and pellets were resuspended in 10 μl distilled water and dropped onto nitrocellulose membranes. β-Galactosidase activity was detected by using a filter assay as described by Breeden & Nasmyth (1985).

RESULTS

Genetic screen for mutants with elevated levels of basal thermotolerance during the exponential phase of growth

To learn more about the mechanisms that coordinate the regulation of basal thermotolerance during the different
growth phases in liquid cultures at 25 °C, we decided to carry out a systematic search for mutants that, in the exponential phase, displayed high basal thermotolerance (see Methods). Fourteen independent mutants were isolated and assembled in six complementation groups (data not shown). Here, we describe the characterization of two of these mutant strains: ScIBT2 and ScIBT3. Both ScIBT2 and ScIBT3 were several thousand times more resistant to a 20 min treatment at 50 °C and formed smaller colonies than their wild-type progenitor, strain W303a-LEU<sup>+</sup> (data not shown).

Genetic analysis of mutant strains ScIBT2 and ScIBT3 indicated that both high thermotolerance and slow growth were recessive traits and that the two phenotypes were the result of a single, nuclear mutation (data not shown). A cross between JF314 and JF411 (ScIBT2 and ScIBT3 derivatives, respectively) showed that neither colony size nor thermotolerance levels were complemented, suggesting that their mutations were allelic (data not shown).

After three consecutive backcrosses to their progenitor wild-type strain, JF099 (ScIBT2 derivative) and JF411 mutant strains retained the high-therмотolerance and slow-growth phenotypes of the original ScIBT2 and ScIBT3 strains. Their basal thermotolerance was several thousand times the level of the wild-type strain (Table 2). Duplication times of the mutants in batch cultures were twice as long as that of the parental strain (Table 2). Both mutant strains and their progenitors failed to grow in media that contained acetate, galactose or glycerol as the carbon source (data not shown). Both ScIBT2 and ScIBT3 (and their derivatives) grew at 37 °C, but not at 38 °C or above. In contrast, their isogenic wild-type strain (W303a-LEU<sup>+</sup>) was able to grow at 40 °C (data not shown).

**ScIBT2, ScIBT3 and derivative strains are allelic to CDC25**

In order to identify the gene that is responsible for the observed phenotypes, we complemented strain JF099. This strain was transformed with a wild-type genome library and the resulting colonies were screened for wild-type colony size. Eight out of 14,000 transformants showed a large colony size as compared with the mutant strain, but in only seven of them was the colony-size transformant plasmid-dependent (plasmids pJF02–pJF08). All plasmid inserts shared the CDC25, YLR311C and YLR312C loci of chromosome XII (Fig. 1a). Due to the known involvement of the Ras/PKA pathway in the control of cell proliferation and stress resistance (Thevelein & de Winde, 1999), we tested the pRY002 plasmid, which contained only the ORF of CDC25 (see Methods), for complementation. As shown in Table 2, pRY002 fully complemented the growth rate at 25 °C and basal thermotolerance of strains JF099 and JF411. We refer to the mutant alleles of CDC25 in ScIBT2 and ScIBT3 and their derivatives as *cdc25-21* and *cdc25-22*, respectively.

We also carried out a complementation analysis of diploid strains that resulted from the cross between *cdc25-21* (JF335) or *cdc25-22* (JF422) strains and *cdc25-1* (LRA25), a well-characterized *cdc25* allele (Petitjean et al., 1990). Diploids were tested for duplication time and thermostolerance. Diploid strains JF2014 (*cdc25-21/cdc25-1*) and JF2015 (*cdc25-22/cdc25-1*) showed slow duplication times and elevated basal thermostolerance (data not shown). We conclude that *cdc25-21* and *cdc25-22* mutants are allelic to *cdc25-1*. At 25 °C, *cdc25-5* (LRA26) and *cdc25-10* (LRA24) cells had no growth defects and were not thermostolerant, as shown previously (Petitjean et al., 1990). In contrast, *cdc25-1* cells (strain LRA25) had a slower growth rate and were much more thermostolerant than their isogenic wild-type strain (LRA89) (Table 2).

We cloned the *cdc25-21* and *cdc25-22* alleles by the allele-rescue procedure (see Methods). The *cdc25-21* allele showed a single-base deletion within the *CDC25* ORF (adenine 2926). This deletion caused a change in the ORF that resulted in a stop codon 36 nt downstream, plus several other stop codons further downstream. Thus, mutant *cdc25-21* is predicted to encode a fusion protein that contains the N-terminal 977 aa of Cdc25p fused to 12 non-native amino acids at the C-terminus (Fig. 1b). In *cdc25-22*, we found an A→C change at position 4088 of the *CDC25* ORF, which changed a histidine to a proline at position 1363 of Cdc25p (Fig. 1b). To determine whether we had characterized the correct mutant gene, we recreated a deletion strain resembling *cdc25-21*, where the C-terminal

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**Table 2. Basal thermostolerance and growth rate of *cdc25* mutants during the exponential phase at 25 °C in SD medium**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Duplication time (h)</th>
<th>Thermotolerance† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-6B (pRS413)</td>
<td>2.65 ± 0.07</td>
<td>0.015 ± 0.01</td>
</tr>
<tr>
<td>W303-6B (pRY002)</td>
<td>2.59 ± 0.11</td>
<td>0.040 ± 0.04</td>
</tr>
<tr>
<td>JF099 (pRS413)</td>
<td>5.66 ± 0.17</td>
<td>61.67 ± 7.07</td>
</tr>
<tr>
<td>JF099 (pRY002)</td>
<td>2.37 ± 0.06</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>JF411 (pRS413)</td>
<td>5.57 ± 0.06</td>
<td>73.72 ± 1.96</td>
</tr>
<tr>
<td>JF411 (pRY002)</td>
<td>2.27 ± 0.02</td>
<td>0.039 ± 0.03</td>
</tr>
<tr>
<td>JF3005 (pRS413)</td>
<td>5.24 ± 0.099</td>
<td>64.19 ± 8.48</td>
</tr>
<tr>
<td>JF3005 (pRY002)</td>
<td>2.64 ± 0.10</td>
<td>0.132 ± 0.01</td>
</tr>
<tr>
<td>RY008</td>
<td>1.90 ± 0.05</td>
<td>0.007 ± 0.01</td>
</tr>
<tr>
<td>LM004</td>
<td>1.90 ± 0.06</td>
<td>0.006 ± 0.005</td>
</tr>
<tr>
<td>LM005</td>
<td>1.95 ± 0.05</td>
<td>0.011 ± 0.003</td>
</tr>
<tr>
<td>LRA89</td>
<td>2.71 ± 0.15</td>
<td>0.032 ± 0.017</td>
</tr>
<tr>
<td>LRA25</td>
<td>3.5 ± 0.02</td>
<td>69.05 ± 5.15</td>
</tr>
</tbody>
</table>

*Relevant genotypes of strains used were: W303-6B (CDC25); JF099 (cdc25-21); JF411 (cdc25-22); JF3005 (Δcdc25); RY008 (CDC25/ CDC25); LM004 (cdc25-21/CDC25); LM005 (cdc25-22/CDC25); LRA89 (CDC25); LRA25 (cdc25-1).

†Survival (%) to heat shock at 50 °C for 20 min during exponential-phase growth at 25 °C. All values are means ± SD of at least three independent experiments.
600 aa of Cdc25 was deleted by insertion of a loxP–kanMX–loxP gene-disruption cassette (strain JF3210). As for cdc25-21 cells, strain JF3210 was highly thermotolerant and had a growth rate twice as long as that of its isogenic wild-type strain (data not shown).

According to the literature, CDC25 is an essential gene and the catalytic C-terminus of Cdc25p is sufficient for viability (Munder et al., 1988; Lai et al., 1993). To exclude the possibility of a mistake in the molecular characterization of the cdc25-21 mutant, we disrupted the CDC25 gene in the W303, BY4743 and LRA wild-type diploid backgrounds by integration of a loxP–kanMX–loxP gene-disruption cassette. Selected diploids were sporulated and asci were dissected in YPDA medium. For RY008 (W303 background), the segregation pattern was 2 : 2 (large vs small colonies) and geneticin resistance always co-segregated with small colony size (Fig. 2a). In the BY4743 and LRA backgrounds, segregation was 2 : 0 (Fig. 2b, c) and no geneticin resistance was found in the viable spores. The correct insertion of the cassette in the W303 tetrads was verified by PCR and sequence analysis (data not shown). Basal thermotolerance and growth rate of two small colonies of a tetrad from the W303 background were identical to those of mutant cdc25-21, whilst the two large colonies were wild-type for both phenotypes (data not shown). These data indicate that, in the W303 background, CDC25 is dispensable for growth in glucose, and confirm earlier reports regarding the lethality of CDC25 deletion in other yeast strains. Strain JF3005 (Δcdc25) was fully complemented by plasmid pRY002 (Table 2).

Similarly to cdc25-21 and cdc25-22 mutant cells, growth of strain JF3005 (Δcdc25) was temperature-sensitive, as they were able to grow at 35 °C, but not at 36 °C. When any of these three cdc25 mutants was transformed with pJF08 or pRY002 (which carry a wild-type CDC25 allele), their growth rate at 40 °C was slower than that of the isogenic wild-type strain (data not shown). This apparent partial complementation of growth rate was attributed to the
elevated plasmid loss (up to 40%) that occurs under these growth conditions (data not shown). Plasmid loss should have a major impact on the growth rate of cdc25 cells that are transformed with plasmids containing CDC25, as cdc25 cells do not grow under these conditions. cdc25-21, cdc25-22 and Δcdc25 mutants that were transformed with plasmids containing CDC25 fully complemented growth in acetate as the carbon source (data not shown).

cdc25-21 does not increase cAMP content upon glucose addition

As Cdc25p is postulated to be an upstream activator of cAMP synthesis (Broek et al., 1987) and Δcdc25 cells are capable of growth in glucose media at 25°C, we asked whether they were able to induce the levels of cAMP in response to glucose addition in glucose-deprived cells. The JF099 strain (cdc25-21) transformed with YCp50 or with pJF08 (carrying the wild-type CDC25 locus) showed no response, whereas the isogenic wild-type strain W303-6B transformed with YCp50::LEU2 showed a rapid increase in cAMP levels in response to glucose addition (Fig. 3). This result suggests that Cdc25p is necessary for the glucose-induced response and reveals that, when expressed in a low-copy plasmid, cdc25-21 behaves as a dominant-negative mutant allele.

Mutants cdc25-21 and cdc25-22 show some stationary-phase properties

Previously described temperature-sensitive cdc25 mutants show some stationary-phase properties, such as glycogen accumulation during the exponential phase of growth (Petitjean et al., 1990) or cell-wall resistance to glusulase digestion at the restrictive temperature (Martegani et al., 1984). To assess whether cdc25-21 and cdc25-22 cells show stationary-phase features other than high heat shock-resistance levels at the optimal temperature, we estimated the levels of glycogen accumulation by iodine staining. Both cdc25-21 (JF099) and cdc25-22 (JF411) mutants hyper-accumulated glycogen when compared with the isogenic wild-type strain (data not shown). Glycogen hyperaccumulation was also a recessive trait, as shown by analysis of heterozygous strains LM004 (cdc25-21/CDC25), LM005 (cdc25-22/CDC25) and LM002 (cdc25-1/CDC25) (data not shown).

Fig. 3. Addition of glucose to derepressed cdc25-21 cells does not trigger a rapid increase in cAMP levels. cAMP levels were measured in response to glucose addition after carbon deprivation in JF099 (pJF08) (■) or JF099 (YCp50) (○) mutant cells or in the isogenic wild-type strain W303-6B (YCp50::LEU2) (●).

Stationary-phase cells also show high resistance to stresses other than heat shock. We tested for resistance to ionic, oxidative and osmotic stress in cdc25-21 (JF099), cdc25-22 (JF411), Δcdc25 (JF3005) and cdc25-1 (LRA25) mutants at 25°C during exponential growth. All four mutants were more tolerant to these stresses when compared with their isogenic wild-type strains (Fig. 4 and data not shown). High resistance to 0·4 M LiCl and 3·5 M sorbitol was also a recessive trait. In contrast, tolerance to oxidative stress by H2O2 treatment was semidominant in all four mutants (Fig. 4 and data not shown). In order to further understand the elevated stress resistance of the cdc25 mutants, we assessed the levels of HSP104, TPS1 (trehalose phosphate synthase), CTT1 (cytosolic catalase T) and GPD1 (glycerol-3-phosphate dehydrogenase) RNAs in the four mutant and isogenic wild-type strains during exponential growth at 25°C. Expression of these genes in wild-type strains is induced by heat, oxidative and osmotic stress (Wieser et al., 1991; Albertyn et al., 1994; Hazell et al., 1995; Lindquist & Kim, 1996). Levels of IPP1 (inosinic pyrophosphatase) were used as a control, as it is not induced by stress (Gasch et al., 2000). In wild-type cells, stress-responsive genes were induced by heat shock and were absent or present in very low amounts at the control temperature of 25°C (Fig. 5 and data not shown). However, all of these genes, with the exception of GPD1 and IPP1, were overexpressed in the four mutant strains at the control temperature and induced further by heat shock (Fig. 5 and data not shown). These results suggest that the high stress resistance of strains cdc25-21, cdc25-22, Δcdc25 and cdc25-1 is due in part to the overexpression of stress-regulated genes. The constitutive expression of stress-inducible genes was a recessive trait (Fig. 5 and data not shown).

An indirect estimate of the structure of the cell wall in the mutants was performed by using a modified cell-wall digestion assay (see Methods). During the exponential phase at 25°C, cells of mutants cdc25-21 (JF099) and cdc25-22 (JF411) showed high resistance to lyticase digestion, which was suppressed by transformation with pJF08 (which contains a wild-type copy of CDC25) (Fig. 6). In contrast, isogenic wild-type cells were very sensitive to lyticase during the exponential phase, whereas in the stationary phase, their cell walls were highly resistant to degradation. To further
confirm these results, electron microscopic observations of the cdc25 mutants were performed to study the structure of their cell wall. The walls of cdc25-21 (JF099) and cdc25-22 (JF410) cells grown at 25°C were thicker than those of cells of the isogenic wild-type strain (Fig. 7). This morphological change was present in both budding and non-budding cdc25-21 and cdc25-22 cells (Fig. 7e, f), supporting the idea that the stationary phase-like phenotypes in these mutants are not exclusive of non-dividing cells (see below). Fluorescence-activated cell sorting (FACS) analyses

![Fig. 4. cdc25 strains are resistant to different stress treatments.](image)

Fig. 4. cdc25 strains are resistant to different stress treatments. Cells were grown to mid-exponential phase and subjected to oxidative (H₂O₂), osmotic (sorbitol) or ionic (LiCl) stress as described in Methods. To assess survival, serial dilutions (1, 1:10, 1:100 and 1:1000 from left to right) were dropped onto YPDA after each stress treatment. Pictures from the haploid mutant strains were taken after 3 days growth, whereas those from the isogenic wild-type haploid and all diploid strains were taken after 2 days growth. Upper panels show haploid strains: CDC25 (W303-6B), cdc25-21 (JF099) and cdc25-22 (JF410). Lower panels show diploid strains: CDC25/CDC25 (RY008), CDC25/cdc25-21 (LM004) and CDC25/cdc25-22 (LM005).

![Fig. 5. Constitutive expression of stress genes in cdc25 mutants during exponential growth in glucose at 25°C is a recessive trait.](image)

Fig. 5. Constitutive expression of stress genes in cdc25 mutants during exponential growth in glucose at 25°C is a recessive trait. Northern blot analysis of transcripts of HSP104, TPS1, CTT1, GPD1 and IPP1 from wild-type CDC25 (W303-6B), cdc25-21 (JF099), cdc25-22 (JF410), CDC25/CDC25 (RY008), CDC25/cdc25-21 (LM004) and CDC25/cdc25-22 (LM005) strains grown to mid-exponential phase in SD at 25°C. IPP1 levels are known to be non-inducible by stress and were used as a control. The lower panel shows an ethidium bromide-stained gel prior to hybridization. Heat-shock treatment was given at 39°C for 15 min.

![Fig. 6. In the exponential phase, the walls of cdc25-21 and cdc25-22 cells are highly resistant to lyticase digestion.](image)

Fig. 6. In the exponential phase, the walls of cdc25-21 and cdc25-22 cells are highly resistant to lyticase digestion. Exponentially grown cells were treated with lyticase and the absorbance of the culture (OD₆₀₀) was measured at different time points. Strains used were wild-type W303-6B (YCp50::LEU2) (●), JF099 (cdc25-21) (○), JF411 (cdc25-22) (▲) and JF099 (pJF08) (■).
also showed that the distribution of cell sizes was shifted towards larger sizes in the mutant strains (data not shown).

**Stationary-phase phenotypes of cdc25-21 and cdc25-22 cells do not require entry into the stationary phase**

The increased duplication time and high thermotolerance of the cdc25-21, cdc25-22 and Δcdc25 mutants can be explained by two different models. In one model, a fraction of the population enters and remains in stationary or G0 phase, while another fraction stays in the cell cycle. In this model, the higher the proportion of cells in G0 or stationary phase, the higher the level of thermotolerance and the duplication time in the culture. In an alternative model, all cells in the population have an extended cell cycle. Both thermotolerance and duplication time are increased, as cells experience a transient arrest in their cell cycle that confers on them a high stress tolerance. Three kinds of experiments were performed to test the validity of either of these two hypotheses: measurement of the ratio of (G0 + G1)/(G2 + M) cells; the effect of gcs1 mutation on growth; and determination of the duplication time in single cells dissected from cultures growing in the exponential phase.

Flow-cytometric analyses of DNA content showed that approximately 61.5 ± 2 % of cells of the cdc25-21 (JF099) strain were in G0 + G1 phase during exponential growth at 25 °C (Fig. 8a). In contrast, isogenic wild-type cells that were grown under the same conditions showed 46 ± 0.6 % of the cells in G0 + G1 (Fig. 8b). Microscopic observations of exponentially growing cultures of cdc25-21 (JF337) cells showed that 77 ± 5 % of the cells were non-budding (G0 + G1), whereas in isogenic wild-type cultures, 57 ± 5 % were non-budding, correlating with the flow-cytometric analyses.
In an effort to distinguish the G1 and G0 phases of the cell cycle, we used a mutation that defines the G0 phase of the cell cycle (gcs1). When stimulated to return to the mitotic cell cycle, single gcs1 mutants show a conditional phenotype of non-proliferation at 14°C if cells have previously entered into the stationary phase (Wang et al., 1996). However, if transferred during the exponential phase from 25 to 14°C, they continue to grow (Drebot et al., 1987). We analysed double cdc25 gcs1 mutants to evaluate whether or not cdc25-21 or cdc25-22 cells enter the stationary phase transiently during exponential growth. Double cdc25 gcs1 mutant strains (JF1061 and JF1080) were grown at 25°C and shifted to 14°C during the exponential or stationary phase. All strains carrying the gcs1 mutation failed to resume growth when transferred to fresh media at 14°C from the stationary phase (data not shown). In contrast, exponential cultures of all strains carrying the gcs1 mutation or the wild-type GCS1 allele growing at 25°C were able to resume growth after transfer to 14°C (Fig. 9). Therefore, the majority of exponentially growing cdc25-21 and cdc25-22 cells should not enter into a transient, stationary phase-like arrest.

If the difference in basal thermotolerance between wild-type and cdc25 mutants is due to cdc25 exponential cultures having a larger proportion of their population in a transient, stationary phase-like arrest (different from G0), a large proportion of single, non-budding cells should display a long lag phase when stimulated to grow on a rich medium. To test this hypothesis, single, non-budding cells were dissected from exponential- or stationary-phase cultures with a micromanipulator and placed onto fresh YPDA plates at 25°C. All cdc25-21 (JF099) and cdc25-22 (JF411) cells from exponential cultures budded 4 h after dissection. All of the isogenic wild-type cells from exponential cultures budded 2 h after dissection. These values were similar to their duplication times in liquid cultures in SD medium during the exponential phase (Table 2). In contrast, all wild-type cells from stationary-phase cultures divided between 4 and 6 h, whereas cdc25-21 and cdc25-22 cells in the stationary phase had a lag period of 14.5 h to accomplish their first cell division. Thus, all cdc25-21 or cdc25-22 exponentially growing cultures are formed by a homogeneous population that divides at the same rate. The unusually long lag period in cdc25-21 or cdc25-22 mutants that exit from stationary phase also suggests that Cdc25p is necessary to exit the G0 phase and to resume the cell cycle. This last observation is in agreement with earlier work (Tatchell, 1993).

**CDC25 is not required for fermentation or respiration**

The fact that cdc25 mutants lose the ability to grow in non-fermentable carbon sources suggested that they were impaired in their respiratory capacity. To test this idea, we analysed their fermentative and respiratory capacity by measuring growth, glucose utilization, oxygen consumption and ethanol production. Glucose was exhausted completely from mutant cdc25-21 (JF099) cultures by about 38 h, whereas isogenic wild-type cultures (W303-6B) took only 27 h to totally consume glucose (Fig. 10a, b). Ethanol production by the wild-type strain reached a maximum of approximately 6.5 g l⁻¹ after about 27 h, corresponding to the glucose exhaustion. Mutant cdc25-21 also produced ethanol concomitantly with glucose depletion, although it
could only produce 4.8 g l\(^{-1}\) after 38 h (Fig. 10c). Once glucose was exhausted, cells of both W303-6B and cdc25-21 entered the post-diauxic phase of growth and ethanol concentration in the media started to diminish (Fig. 10a and c). During exponential growth in YPDA medium, the cdc25-21 mutant consumed oxygen at a lower rate, in agreement with its slower growth rate (Fig. 10d). Addition of potassium cyanide abolished oxygen consumption in both strains (Fig. 10d). These data indicate that loss of Cdc25p function does not impair either fermentation or respiration in glucose media.

**DISCUSSION**

**Cdc25p is dispensable for viability in glucose media and plays an important role for growth under conditions in which the cAMP/PKA pathway is downregulated**

We have isolated two novel mutant alleles of the CDC25 gene of *S. cerevisiae*, cdc25-21 and cdc25-22, that show conspicuous phenotypes at 25°C. Previously described temperature-sensitive cdc25 mutants arrest at G1 at the restrictive temperature (36°C) and, contrary to wild-type cells, remain thermotolerant under sustained incubation at 36°C. At the permissive temperature, temperature-sensitive cdc25 mutants show decreased adenylate cyclase activity, a longer lag phase, decreased phosphatase derepression, accumulation of glycogen during exponential growth, impairment of gluconeogenic growth and, in certain deletion mutants, no sporulation (Iida & Yahara, 1984; Munder *et al*., 1988; Petitjean *et al*., 1990). In addition to some of the previous phenotypes, the cdc25 alleles isolated in this work showed, at 25°C, elevated thermostolerance, resistance to ionic, oxidative and osmotic stress, a slow growth rate and other stationary phase-like characteristics. We observed that the cAMP response to glucose addition of cdc25-21 is dominant-negative to CDC25 when present in a single-copy plasmid (see discussion below). Similarly, resistance to oxidative stress is a semidominant trait. However, expression of *CTT1* and other stress genes was recessive, suggesting that the high resistance to oxidative stress was conferred by a single recessive gene.
damage by H$_2$O$_2$ in the $\text{cdc25-21}$ and $\text{cdc25-22}$ mutants is only partially dependent on $\text{CTT1}$ constitutive expression. Thus, the new $\text{cdc25}$ alleles found in the W303 background are becoming a very important tool to study the function of Cdc25 independently of high temperatures and their G$_1$ arrest-associated effects.

The $\text{cdc25-21}$ allele was predicted to encode a Cdc25p lacking the catalytic, C-terminal domain. The $\text{cdc25-22}$ allele has a histidine→proline substitution at position 1363 within the catalytic domain of Cdc25p. We also demonstrated that $\Delta \text{cdc25}$ cells derived from the W303 strain were viable and identical phenotypically to $\text{cdc25-21}$ and $\text{cdc25-22}$ cells. Therefore, the Cdc25p encoded by $\text{cdc25-21}$ and $\text{cdc25-22}$ mutants most probably lacks the capacity to activate Ras. Earlier reports indicated that the C-terminal moiety of Cdc25p is essential for viability (Munder et al., 1988). Disruptions at a central position of Cdc25p (d3) allow viability, but not gluconeogenic growth or sporulation (Munder et al., 1988). Our data indicate that, in the W303 background, Cdc25p is dispensable for growth in glucose. We also conclude that the C-terminus of Cdc25p is essential for growth in gluconeogenic carbon sources, such as acetate and glycerol. This finding complements previous reports indicating that the N-terminus is necessary for growth in acetate or glycerol media (Munder et al., 1988). As the $\text{cdc25-21}$ mutant contains an intact N-terminus and is not able to grow in acetate or glycerol as sole carbon sources, we propose that the full-length Cdc25p is essential for growth in these media. As mutant $\text{cdc25-21}$ grows during the post-diauxic phase by consuming ethanol and oxygen, we suggest that this growth defect is not due to a respiratory deficiency, but possibly to a gluconeogenic defect. Thus, Cdc25p is dispensable for fermentation or respiration in glucose media. The fact that these $\text{cdc25}$ mutants do not grow in galactose suggests that Cdc25p is required to change from a glucose-repressed state to a 'derepressed' state. $\text{cdc25-21}$ (JF099), $\text{cdc25-22}$ (JF411) or $\Delta \text{cdc25}$ (JF3005) cells transformed with pLA41 (which contains a lacZ-UAS$_{\text{GAL}}$ fusion) showed no detectable β-galactosidase activity during the exponential phase in glucose, implying that, in these cells, the galactose-regulatory system is repressed completely (data not shown). Moreover, when shifted to galactose as the only carbon source, wild-type cells showed the expected enhancement of β-galactosidase activity, whereas mutant cells showed no signs of induction, implying that Cdc25p activity is required for glucose derepression (data not shown). Our data are consistent with previous findings that indicate that overexpression of $\text{CDC25}$ induces derepression of the galactose-regulatory system in the presence of glucose (Van Aelst et al., 1991; Rudoni et al., 2000). We suggest that the unique role of Cdc25p in galactose derepression is non-redundant, as inactivation of its function completely abolished the response.

$\text{SDC25}$, which encodes a protein with homology to the catalytic domain of Cdc25p, was originally described as a multicopy suppressor of temperature-sensitive $\text{cdc25}$ mutants (Damak et al., 1991). This homologue could account for the non-lethal phenotype that is observed in $\Delta \text{cdc25}$ mutants in the W303 background. In other backgrounds, such as S288C, $\text{SDC25}$ is a pseudogene (Saccharomyces Genome Database). In the W303 strain, $\text{SDC25}$ encodes a complete ORF that is suggested to be expressed only in non-fermentable carbon sources and upon glucose exhaustion (Boy-Marcotte et al., 1996). Both $\text{cdc25-21}$ and $\Delta \text{cdc25}$ were able to grow by fermentation in glucose-containing media, suggesting that $\text{SDC25}$ could play an active role during glucose fermentation in the wild-type strain W303. Alternatively, $\text{SDC25}$ expression might be negatively controlled by $\text{CDC25}$. Inactivation of $\text{CDC25}$ could have simply unveiled this relationship.

The transient increase in cAMP in response to glucose was not complemented by the wild-type gene, present in a low-copy plasmid, in the $\text{cdc25-21}$ background. Likewise, resistance to H$_2$O$_2$ by $\text{cdc25-21}$, $\text{cdc25-22}$ and $\text{cdc25-1}$ was semidominant. These dominant and semidominant effects can be explained based on the oligomerization state of Cdc25p. Cdc25p forms dimers (Camus et al., 1997) and its N-terminal domain is capable of dimerizing with the full-length protein, with itself or with the C-terminus (Chen et al., 2000). Cdc25p can also dimerize with Sdc25p (Camus et al., 1997). In a heterozygous strain, wild-type homodimers, mutant homodimers and heterodimers must form. If we assume that only wild-type homodimers are fully functional, then it is reasonable to expect that mutant Cdc25p monomers sequester a fraction of wild-type monomers, thus decreasing the levels of wild-type Cdc25p homodimers in the $\text{CDC25}$/cdc25-21 or $\text{CDC25}$/cdc25-22 strain. Low levels of Cdc25p seem to be sufficient to restore wild-type growth rate and basal thermotolerance at 25°C in glucose, but not to restore the transient elevation of cAMP in response to glucose or sensitivity to H$_2$O$_2$, implying that the full control of these phenomena requires higher Cdc25p activities. The essential role of Cdc25p for growth in non-fermentable carbon sources is similar to that observed for adenylate cyclase and Ras2p (Petitjean et al., 1990). It is conceivable that when the G protein-coupled receptor (GPCR) system is not functional, as in the absence of glucose, there is a need to maintain a minimum concentration of cAMP to support viability.

**cAMP transient response to glucose addition is impaired in $\text{cdc25}$ mutants**

Mutant $\text{cdc25-21}$ is able to grow in glucose media, although it does not show the rapid response to glucose addition after carbon deprivation. We suggest that the GPCR system requires Cdc25p to function properly. Our data confirm earlier results that were obtained by studying the cAMP response of temperature-sensitive $\text{cdc25-5}$ mutants after addition of glucose at 37°C (Van Aelst et al., 1990). By using $\text{cdc25-21}$ cells, we were able to rule out the possible secondary effects of high temperatures in previously described $\text{cdc25-5}$ temperature-sensitive mutants. Interestingly,
the transient response to glucose was not complemented by wild-type CDC25 in the cdc25-21 background, possibly as a result of the formation of defective heterodimers, as discussed above. This finding contrasts with earlier reports that indicated full complementation of the cAMP response in cdc25-5 mutants by CDC25 (Van Aelst et al., 1991).

**cdc25 mutants show stationary-phase properties during the exponential phase**

Our results show that mutants cdc25-21 and cdc25-22 display some stationary-phase properties. Glycogen hyperaccumulation seen in these mutants is in agreement with previous observations by Petitjean et al. (1990). Martegani et al. (1984) observed increased cell-wall resistance to glusulase digestion in a cdc25-1 mutant at the restrictive temperature (36 °C). Our data rule out the possibility that cell-wall resistance to digestion by lyticase is a secondary effect of heat shock or cell-cycle arrest in G₁. Electron micrographs show that cdc25 mutants do indeed have thicker cell walls at the optimal temperature. The fact that dividing cdc25 cells show thicker cell walls suggests that their stationary phase-like properties are not due to entrance to the stationary phase (see below) and that this property is compatible with cell division.

In exponentially growing cultures, most cdc25-21 and cdc25-22 cells are non-budding. This suggests that cdc25-21 and cdc25-22 cells have an extended G₁ phase and/or enter the stationary phase, explaining their slow duplication time. Experiments performed with double cdc25-21 gcs1 and cdc25-22 gcs1 mutants suggest that the slow growth of the single cdc25-21 and cdc25-22 mutants during the exponential phase is not due to a transitory, stationary-phase arrest that is imposed in the whole population. It appears that Cdc25p regulation of basal thermostability and other stationary phase-like features (cell-wall changes, stress-gene expression, glycogen accumulation etc.) during the exponential phase do not require the function of Gcs1p. These findings are in disagreement with experiments performed by Drebot et al. (1990); these authors used the temperaturesensitive cdc25-1 mutant and showed that after 9 h incubation at the restrictive temperature of 37 °C, cells entered the stationary phase. In contrast, we observed that during the exponential phase, double cdc25-21 gcs1, cdc25-22 gcs1 or single gcs1 mutant cells, arrested by heat shock at 39 °C, resumed growth immediately after incubation at 14 °C (data not shown). Our results indicate that a conditioning heat shock is neither necessary nor sufficient to impose the stationary-phase arrest. It may be possible that this effect is allele-specific and therefore the cdc25-1 mutation is revealing a new activity for Cdc25p. Alternatively, the conditional phenotype in the double mutant gcs1 sed1 that was studied by Drebot et al. (1990) may have caused the mentioned effect. If that is the case, we suggest that Sed1p acts synergistically with Cdc25p in controlling entry into the stationary phase. Microscopic observation of individual, non-budded cells from exponentially growing cultures showed that they all divide at the same rate, supporting the idea that cdc25-21 and cdc25-22 cells do not have subpopulations of non-dividing cells in the stationary phase.

The above results suggest that all exponentially growing cdc25-21 and cdc25-22 cells spend a longer time in the G₁ phase. This extended period in the G₁ phase allows them to follow a distinct developmental programme (i.e. elevated stress resistance, glycogen hyperaccumulation, changes in cell-wall composition and upregulation of stress genes). Alternatively, these stationary phase-like characteristics may be defining a Gsc1p-independent and transitory arrest in the cell cycle that is different from G₀ from stationary phase and from G₁.

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