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Kluyveromyces marxianus flocculence and growth at high temperature is dependent on the presence of the protein p37

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A *Kluyveromyces marxianus* mutant deficient in p37, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-like protein, was obtained and characterized with respect to flocculation behaviour, resistance to temperatures above the optimum for growth, morphology, growth, calcofluor white sensitivity and GAPDH activity. In YPD media, the mutant cells were unable to flocculate and were thermosensitive. However, this thermosensitivity could be overcome by the presence of calcium. Calcofluor white was toxic to the mutant, indicating that the mutation affects cell wall structure. The contribution of p37 to total GAPDH activity was 25% when cells were using glucose as carbon source and 50% when cells were growing in 3% ethanol. These results indicate that p37 is likely to be involved in thermotolerance and flocculation, which can be related to its contribution to cell wall integrity.

Keywords: thermotolerance, flocculation, *Kluyveromyces marxianus*, cell wall proteins

INTRODUCTION

Yeast flocculation results from the aggregation of hundreds or thousands of cells due to interactions between components of the cell wall surface. The nature of these cell interactions has been elucidated by several approaches, for example protein synthesis inhibition by cycloheximide (Nishihara *et al.*, 1976) and proteinase K digestion (Nishihara *et al.*, 1977), indicating the involvement of proteins (Moradas-Ferreira *et al.*, 1994). In 1982, Miki *et al.* proposed a tentative model in which flocculation results from interactions between lectins and carbohydrates on the surface of the cell walls. More data have since become available that support this model (Stratford & Carter, 1993).

Kluyveromyces marxianus flocculation is concomitant with the accumulation of a protein (p37) in the wall of flocculent cells. Indeed, the p37 concentration in the cell wall increases to 11% of the total wall proteins released by SDS, suggesting that it is involved in the interaction between cells leading to flocculation (Fernandes *et al.*, 1992, 1993). Although it is well established that cell wall proteins are responsible for flocculation and different putative flocculins have been identified in *Saccharomyces cerevisiae* (Stewart *et al.*, 1976; Holmberg, 1978) and *Hansenula anomala* (Saito *et al.*, 1990), definitive proof for such a role has only been presented for proteins encoded by the *Flo* genes in *S. cerevisiae* (Stewart & Russell, 1977; Russell *et al.*, 1980; Vezinhet *et al.*, 1991; Teunissen, *et al.*, 1993). Brewer's yeast flocculence has been related to an increase in cell surface hydrophobicity, apparently induced by growth limitation, and correlated with the presence of large numbers of bundles of long fimbriae-like structures on the cell surface (Straver *et al.*, 1993).

Purification and characterization of *K. marxianus* p37 revealed a high identity to cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Subsequently, the gene encoding p37, *GAP1*, was cloned, sequenced and identified as a member of the GAPDH multigenic family of *K. marxianus*, which consists of three genes: *GAP1* (encoding p37), *GAP2* and *GAP3*. The coding region of these genes has high homology: the identity between *GAP2* and *GAP3* is almost 99·6%, and between these two genes and *GAP1* is 86% (Fernandes *et al.*, 1995). In contrast, the homology between the known flanking regions of *GAP1* and the flanking regions of *GAP2* and *GAP3* is lower (55%). There is differential expression of the members of the GAPDH family: in the presence of glucose, *GAP1* and *GAP2* mRNA synthesis is observed but no *GAP3* transcripts can be detected; using a non-

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

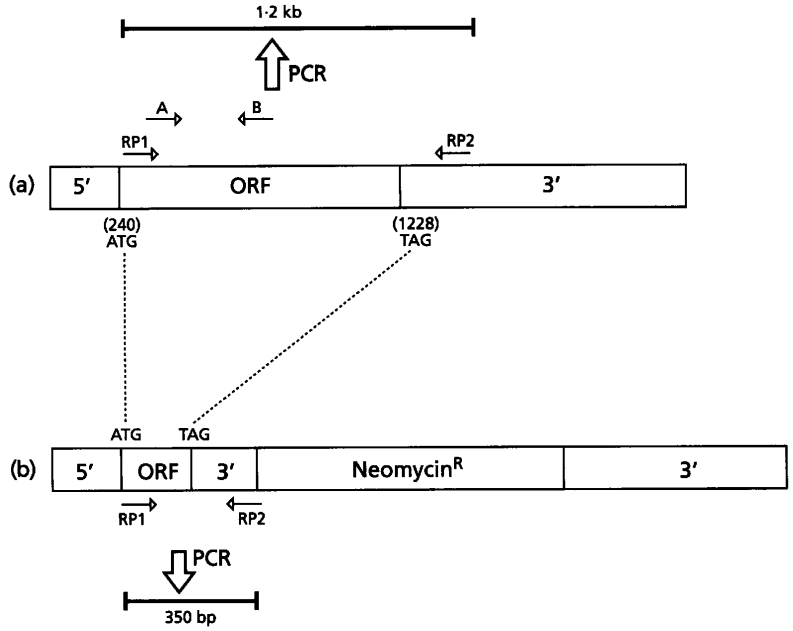

Fig. 1. *GAP1* disruption by excision of part of the coding region and introduction of the neomycin resistance gene. (a) PCR amplification of the *GAP1* coding region using RP primers results in PCR products of 1·2 kb. The positions of primers A and B are shown. (b) PCR amplification of the disrupted *GAP1* gene using RP primers results in PCR products of 350 bp. 3′, 3′ flanking region; 5′, 5′ flanking region.

fermentable carbon source such as ethanol, the transcription of the *GAP2* gene is strongly reduced, in contrast to the transcription activation of *GAP1* (Fernandes *et al.*, 1995).

Since p37 is present on the cell surface, a signal peptide was expected to be found in its amino-terminal end. However, gene sequence analysis did not show any sequence coding for a hydrophobic peptide that could be identified as a signal for protein translocation to the endoplasmatic reticulum. Therefore, this translocation could not be mediated by the SRP (signal recognition particle); thus it must utilize another mechanism, as also reported for 3-phosphoglycerate kinase, another glycolytic enzyme (Alloush *et al.*, 1997).

In the present work, aimed at evaluating the role of p37 in the cell wall and its correlation with flocculation, a *K. marxianus* mutant deficient in p37 was obtained by *GAP1* disruption. The mutant cells were no longer able to flocculate and became temperature-sensitive. This could be related to changes in cell wall stucture, as suggested by the sensitivity of the mutants to calcofluor white.

METHODS

Cell growth conditions. *K. marxianus* ATCC 10022 cells were grown in YPD (2% glucose, 2% Bactopeptone and 1% yeast extract) or YP supplemented with ethanol as a carbon source (2% Bactopeptone, 1% yeast extract and 3% ethanol) at 26 °C or 40 °C.

***GAP1* disruption.** This was performed using the 'one-step gene disruption method' (Rothstein, 1983). Part of the coding region between base 353 and base 1205 was excised by digestion with *Sal*I. The neomycin resistance gene was introduced in the 3′ flanking region of the disrupted gene (Fig. 1). A PCR amplification product of this fragment, including the 240 bp 5′ flanking region and 800 bp of 3′ flanking region, was used to transform *K. marxianus* ATCC 10022 cells.

Yeast transformation. *K. marxianus* cells were transformed by the spheroplast transformation method (Ausubel *et al.*, 1987).

PCR. This was performed using 500 ng genomic DNA and PCR primers RP1 (5′ AAAGGATCCAGATGGTTTCTATTGCTAT 3′) and RP2 (5′ AACGGATCCCTAAGACCTGAAAGACATT 3′). A final volume of 50 µl was used and the conditions were as follows: 1 mM of each primer, 200 mM Tris/HCl (pH 8·4) 500 mM KCl, 7 mM $MgCl_2$, 200 mM dNTPs. One unit of *Taq* polymerase (Gibco BRL) was added after an initial denaturation step of 10 min at 94 °C, followed by 30 cycles of 94 °C for 40 s, 60 °C for 1 min and 72 °C for 1 min. The PCR products were analysed on a 0·8% agarose gel.

Southern blot and hybridization. Aliquots (20 µl) of *K. marxianus* ATCC 10022 wild-type and *gap1* mutant genomic DNA were digested with *Hin*dIII. The digested DNA was separated in a 0·8% agarose gel and transferred onto Hybond-N nylon membrane (Amersham) under alkaline conditions (Sambrook *et al.*, 1989). The DNA was covalently bound to the membrane by UV irradiation and subsequently pre-hybridized for 1 h at 65 °C with 6 × SSC, 5 × Denhardt's solution, 0·5% SDS and 20 mg herring sperm DNA ml^{-1}. The blot was hybridized in the same solution at 65 °C for 16 h using as probe the ^{32}P-labelled PCR fragment obtained from the amplification of the initial part (350 bp) of the coding region of *GAP1* with primers A (5′ ATTCGCTCATTCAACTGGTTTCGG 3′) and B (5′ AGGCAGTCAG ATGATGCTTTTATG 3′) (the positions of primers A and B are shown in Fig. 1), and using as template 25 ng of the plasmid pGAPKm1 (containing the coding region and part of the flanking regions of *GAP1*).

Cell growth. Cells were inoculated into YPD or YPD supplemented with $CaCl_2$ in an Erlenmeyer flask with five times the capacity of the volume of cell culture used. The cell cultures were incubated with shaking at 26 °C or 40 °C. The

optical density at 600 nm was determined each hour to evaluate cell growth.

Quantification of biomass. Aliquots (50 ml) of cell culture were filtered under vacuum using a pre-weighed filter (ME 25/41 ST membrane filter, Schleicher & Schuell). The cells were washed twice with sterile water and the filter was dried at 80 °C for 24 h. The filter was cooled in a dessicator and weighed to determine the amount of biomass present, expressed in g l^{-1}.

Quantification of glucose and ethanol in cell cultures. Samples (2 ml) of culture were taken and centrifuged at 16000 *g* for 2 min. The supernatants were stored at −20 °C until use. Ethanol was quantified using the ethanol UV test kit from Boehringer Mannheim. Glucose was quantified by the glucose oxidase/peroxidase method: 2·5 ml TGO reagent [0·5 M Tris/HCl (pH 7), 20 U glucose oxidase ml^{-1}, 0·38 U peroxidase ml^{-1}, 0·05 mg *o*-dianisidine hydrochloride ml^{-1}, 1% Triton X-100] was added to 0·5 ml of sample, the mixture was incubated for 20 min at 37 °C and the absorbance at 420 nm was determined. Glucose was estimated by reference to a standard curve prepared with known amounts of glucose.

Calcofluor white sensitivity tests. These were performed according to the method described by Ram *et al.* (1994), with the following modifications: cells were incubated in 5 ml YPD medium and incubated at 26 °C until an OD_{640} of 8 was reached. Dilutions of 10, 100, 1000 and 10000 times were prepared and 5 µl of each spotted on a YPD plate containing 50 µg calcofluor white ml^{-1}. The plates were incubated at 26 °C and growth was evaluated after 2 d incubation (F. M. Klis, personal communication)

Enzyme assays. These were performed using the method described by Holland & Westhead (1973).

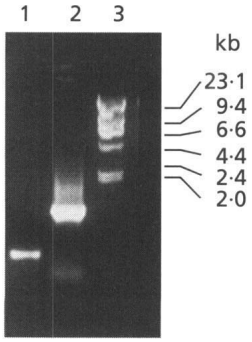

Fig. 2. PCR products obtained using RP primers designed for the amplification of the *GAP1* coding region. Lanes: 1, *gap1* mutant; 2, wild-type; 3, *Hin*dIII-digested λ DNA size markers.

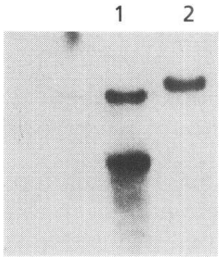

Fig. 3. Southern analysis of genomic DNA for the identification of the coding region of the three GAPDH genes of *K. marxianus*. Lanes: 1, wild-type; 2, *gap1* mutant.

RESULTS

Disruption of the *GAP1* gene

To determine whether *K. marxianus* flocculation is dependent on the presence of p37, a *gap1* mutant was produced by homologous recombination (Rothstein, 1983). This was performed by the introduction into *K. marxianus* ATCC 10022 of a DNA fragment of 3448 bp, obtained by PCR amplification of the disrupted gene. Two hundred and forty base pairs of *GAP1* 5′ flanking region and 800 bp of its 3′ flanking region were included in the PCR product used to transform the *K. marxianus* cells. This approach guaranteed that the homologous recombination could only occur between the flanking regions, aiming the specificity in the targeting of the mutation to *GAP1*, since the flanking regions, in contrast to the coding regions, are not homologous between the different members of the GAPDH gene family of *K. marxianus*.

Analysis of genomic DNA

To confirm the mutation of the *GAP1* gene in the yeast genome, genomic DNA was prepared from either *K. marxianus* wild-type or transformed cells and screened by PCR and Southern blotting.

PCR. When the PCR products from the *gap1* mutant obtained using primers (RP1 and RP2) designed for amplification of part of the *GAP1* coding region were separated on an agarose gel (0·8%), a DNA fragment of the expected size, 350 bp, was revealed, whilst the PCR product from the wild-type was 1·2 kb (Figs 1, 2).

Southern blotting. *K. marxianus* genomic DNA from wild-type and *gap1* mutant cells was digested with *Hin*dIII and hybridized with a ^{32}P-labelled probe specific for the coding region of the three *K. marxianus* GAPDH family genes. As reported previously (Fernandes *et al.*, 1995), a DNA fragment of 8·0 kb due to the presence of *GAP2* and *GAP3* and one band of 4·0 kb due to the presence of *GAP1* should be obtained for wild-type cells. In fact, the two expected fragments were observed for wild-type *K. marxianus*, but only the 8·0 kb fragment was obtained for the mutant cells, indicating the absence of the intact *GAP1* gene (Fig. 3).

Characterization of the mutant

With the aim of evaluating the effects of *GAP1* disruption in *K. marxianus*, the mutant cells were characterized with respect to morphology, flocculation behaviour, growth, biomass, metabolism, calcofluor white sensitivity and GAPDH activity.

Morphology. Morphological analysis of the *gap1* mutant by light microscopy showed that the (*gap1*) mutant cells

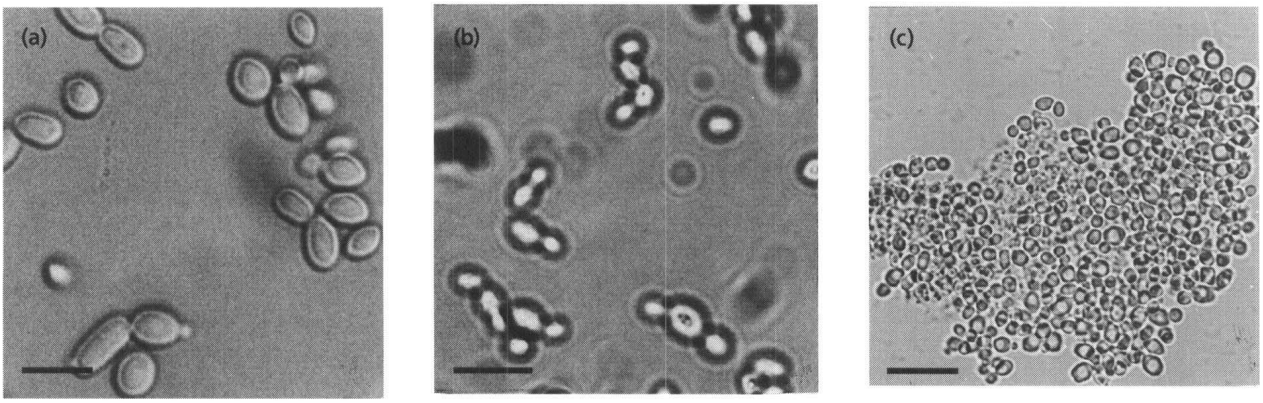

Fig. 4. (a) *K. marxianus* wild-type cells grown in YPD at 26 °C. Bar, 6 μm. (b) *gap1* mutant cells grown in YPD at 26 °C. Bar, 6 μm. (c) Flocculated *K. marxianus* wild-type cells grown in YPD medium containing 200 mM $CaCl_2$ at 40 °C. Bar, 15 μm.

were smaller and looked spherical, in contrast to the elongated appearance of wild-type cells (Fig. 4a, b).

Flocculation behaviour. Flocculation tests revealed that the mutant cells were no longer able to flocculate when growing at 40 °C in the presence of calcium as described for wild-type cells (Fernandes *et al.*, 1993) (Fig. 4c).

Cell growth. This was evaluated at different temperatures, with carbon sources and in the presence of $CaCl_2$. The results indicated that in YPD and at 26 °C, growth of the mutant cells was different from that of wild-type *K. marxianus*. The *gap1* mutant had a much longer lag phase, followed by an exponential phase that led to a considerably higher final OD_{600} (Fig. 5a). When ethanol was used as the carbon source, the growth curve of the mutant differed from that of the wild-type, again leading to a higher OD_{600} (Fig. 5b). With regard to the effect of temperature on growth, it was verified that mutant cells were sensitive to an increase in temperature. Indeed, incubation of mutant cells at 40 °C caused death, in contrast to wild-type cells, which were not affected (Fig. 5c). If calcium (200 mM) was added to the culture medium of mutant cells, growth was rescued, although with a lower yield (Fig. 5c). It was verified that the mutant growth rate at 40 °C was dependent on the calcium concentration; at increasing calcium concentrations (50, 100 and 150 mM) growth increased proportionally (data not shown).

Biomass, glucose uptake and ethanol production. The results obtained concerning biomass showed that the dry weight values of the *gap1* mutant cells were lower (about 15% less) than those of wild-type cells at equivalent OD_{600} values. Glucose uptake and ethanol production of mutant and wild-type *K. marxianus* cells were also different; in fact, the rate of glucose consumption was relatively higher in the *gap1* mutant, and ethanol was produced in greater amounts (Fig. 6a, b). The ratio between the amount of biomass produced and quantity of substrate consumed was calculated for both mutant and wild-type cells. A lower value (about 0·14) was obtained for the *gap1* mutant, compared to that (about 0·20) obtained for the wild-type cells.

Calcofluor white sensitivity. Calcofluor white is a negatively charged fluorescent dye that is used to detect cell wall mutants because it interferes with cell wall assembly. Cells with a weakened cell wall might not be able to withstand the additional disturbance of the cell wall caused by such a drug, resulting in lethality at concentrations that are not lethal to wild-type cells (Ram *et al.*, 1994). Fig. 7 shows the effect of calcofluor white on *gap1* mutant cells (Fig. 7b ii) compared to its effects on wild-type *K. marxianus* cells (Fig. 7a ii).

Contribution to total GAPDH activity. The contribution of the *GAP1* gene encoding p37 to total GAPDH activity was about 25% when cells were using glucose as carbon source and 50% in 3% ethanol (Table 1).

DISCUSSION

In previous studies on the flocculation of *K. marxianus* it was revealed that this yeast accumulates a small glycoprotein with an apparent molecular mass of 37 kDa – p37 – concomitant with the ability to flocculate (Fernandes *et al.*, 1993). This protein is encoded by a member of a GAPDH family named *GAP1* which has high homology with the other two members of the family. However, in contrast to the other GAPDH genes, *GAP1* expression was increased when ethanol was used as a carbon source. With the aim of elucidating the role of the p37 in flocculation behaviour, a *gap1* mutant was obtained and characterized.

To this end, disruption of the *GAP1* gene was achieved by removing a large portion of the coding region and inserting the neomycin resistance gene, a strategy that made use of the differences in the sequence of the flanking regions between *GAP1*, *GAP2* and *GAP3*. The disruption of *GAP1* was verified both by analysis of the

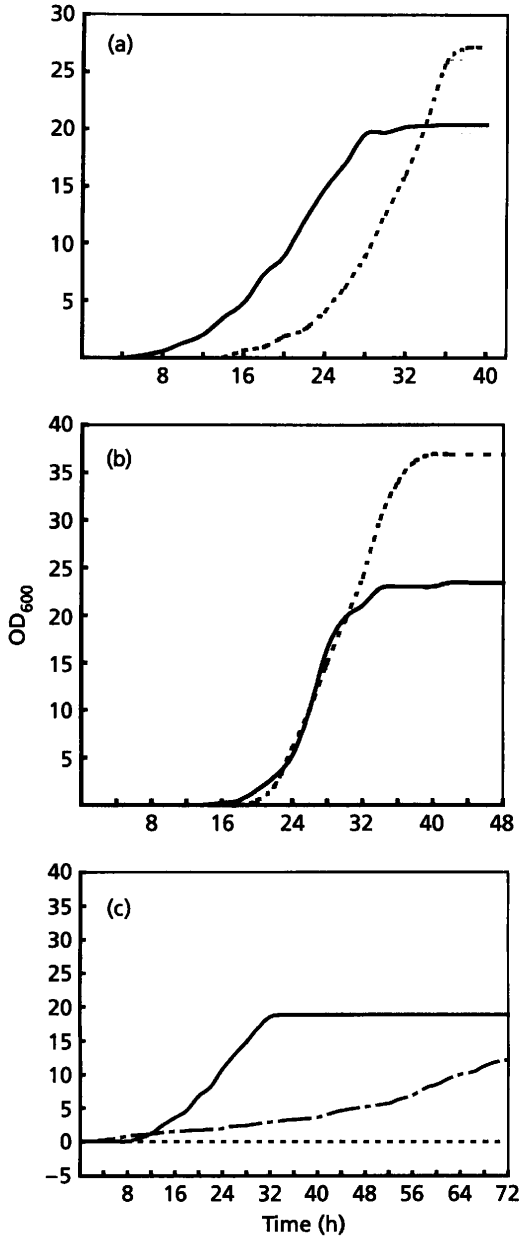

Fig. 5. (a) Growth of *K. marxianus* wild-type (——) and *gap1* mutant (– – –) cells in YPD medium at 26 °C. (b) Growth of *K. marxianus* wild-type (——) and *gap1* mutant (– – –) cells in YP medium supplemented with ethanol (3%) at 26 °C. (c) Growth of wild-type *K. marxianus* cells in YPD medium at 40 °C (——), and *gap1* mutant cells in YPD medium (– – –) and YPD supplemented with 200 mM $CaCl_2$ (— – —) at 40 °C.

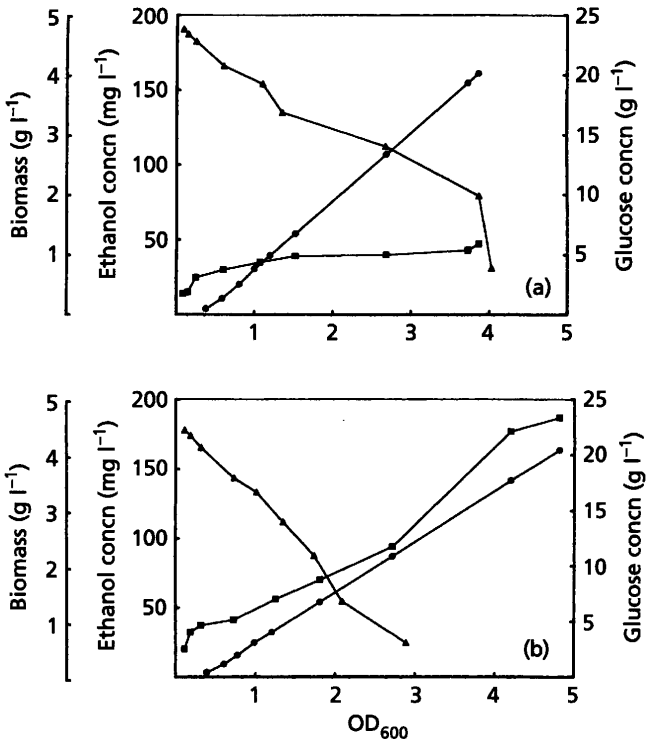

Fig. 6. Biomass (●), glucose consumption (▲) and ethanol production (■) by (a) wild-type *K. marxianus* cells and (b) *gap1* mutant cells.

DNA fragments obtained by PCR using specific primers (Fig. 2) and by Southern analysis (Fig. 3), and both assays led to the conclusion that the mutation of *GAP1* had been successful.

The mutant cells exhibited a different morphology (Fig. 4a, b) when compared with the wild-type: they were smaller in size and they had a spherical instead of a more elongated shape. This altered morphology can be a natural consequence of genetic manipulation of the yeast as has been reported by Veenhuis *et al.* (1978); Wright *et al.* (1988); Binder *et al.* (1991); Simon *et al.* (1992); and Vergères *et al.* (1993).

The correlation between the expression of the *GAP1* gene and the subsequent presence of the protein in the cell wall with the flocculation behaviour of the *K. marxianus* strain is clear as the mutant cells were no longer able to flocculate in the presence of calcium as occurs with the wild-type. Additionally, the mutant was sensitive to calcofluor white (Fig. 7a, b), which indicates that the structure of the cell wall is affected (Ram *et al.*, 1994).

Besides the loss of flocculation, the disruption of *GAP1* led to other changes such as those in growth and temperature tolerance. When *gap1* cells were growing at 26 °C they exhibited a more prolonged exponential phase and cell density reached a higher value (Fig. 5a); however, due to a change in cell size, the biomass yield remained the same (Fig. 6). With regard to the fermentation yield, when glucose and ethanol were estimated, it was found that the mutant cells were able to produce more ethanol. The shift to a more fermentative metabolism can be related to the decrease in GAPDH activity (Table 1). In fact, since *GAP1* contributes to GAPDH activity, its disruption probably affects the rate of production of energy and reducing equivalents by the glycolytic pathway. As a consequence, the cells change to a more fermentative metabolism, which is a less

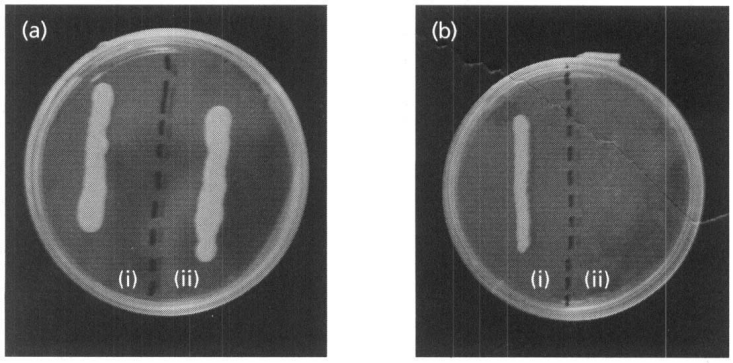

Fig. 7. Wild-type (a) and *gap1* mutant (b) *K. marxianus* cells grown on YPD agar (i) and YPD agar supplemented with calcofluor white (ii).

Table 1. Assay of GAPDH activity in *K. marxianus* strains using different carbon sources

Strain	Growth medium	GAPDH activity [min^{-1} (mg total protein)$^{-1}$]
Wild-type	2% glucose	2·0
gap1 mutant	2% glucose	1·5
Wild-type	3% ethanol	1·4
gap1 mutant	3% ethanol	0·7

efficient but quicker way of obtaining energy for cell survival. In the wild-type, the contribution of p37 to total GAPDH activity was not negligible as it represented 25% when glucose was utilized as carbon source. When ethanol was used as the carbon source, it contributed 50% of total activity. This is in agreement with a previous finding that *GAP1* expression is increased when cells are growing in ethanol, the expression of *GAP2* being very reduced (Fernandes *et al.*, 1995). In the presence of ethanol, this high GAPDH activity cannot be related to the rate of glycolysis, suggesting an additional role for the protein. In fact, data from different cell systems revealed that it has many different cellular localizations and functions. Recent evidence revealed the presence of GAPDH in the surface of *Candida albicans* (Gil-Navarro *et al.*, 1997) and previously GAPDH has been found to be associated with the red blood cell membrane (Allen *et al.*, 1987). Its mutation alters endocytosis in CHO cells (Robbins *et al.*, 1995); it is involved in multiple binding activity in group A streptococci (Pancholi & Fischetti, 1992); in bundling and unbundling of miocrotubules (Huitorel & Pantolini, 1985); protein kinase activity in the phosphorylation of transverse-tubule proteins (Kawamoto & Caswell, 1986); ssDNA binding activity (Perucho *et al.*, 1977); tRNA binding activity (Singh & Green, 1993) and uracil DNA glycosylase activity (Meyer-Siegler *et al.*, 1991).

K. marxianus is quite tolerant of temperatures up to 42 °C (Sá Correia & van Uden, 1982; Vivier *et al.*, 1993), and the mutant strain grows well at 40 °C provided that calcium is present. Indeed, the *gap1* mutant cannot grow at 40 °C in the absence of calcium and cell death is observed (Fig. 5c). The addition of calcium after a 24 h period at 40 °C cannot rescue cell growth. A relationship between thermosensitivity and the ability of the cells to flocculate was also reported for *S. cerevisiae*, which becomes more thermoresistant when the cells are able to flocculate (Bossier *et al.*, 1997). At present we cannot put forward any mechanisms that could account for the effect of calcium on thermotolerance; however, it is plausible to envisage that, by interacting with the negative charges on the surface, calcium can stabilize the structure of cell walls affected by the absence of p37, allowing the cells to avoid cell disruption and survive.

The present work provides evidence that p37 plays a key role in the architecture of the cell wall in conditions where flocculation occurs as there is a net correlation between the presence of p37 with the ability of cells to flocculate, and in thermotolerance as mutant cells are no longer able to grow at a more elevated temperature. Flocculation of *K. marxianus* is induced by a temperature upshift from 26 °C to 40 °C (Fernandes *et al.*, 1993); therefore p37 is probably related to a cell protection mechanism against thermal stress, which leads to flocculation. In fact, flocculation has already been described as a defensive mechanism developed by cells to survive under stress (Moradas-Ferreira *et al.*, 1994).

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