KIHsl1 is a component of glycerol response pathways in the milk yeast *Kluyveromyces lactis*

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In *Saccharomyces cerevisiae*, *HSL1* (*NIK1*) encodes a serine-threonine protein kinase involved in cell cycle control and morphogenesis. Deletion of its putative orthologue in *Kluyveromyces lactis*, *KIHSL1*, gives rise to sensitivity to the respiratory inhibitor antimycin A (AA). Resistance to AA on glucose (Rag^+ phenotype) is associated with genes (*RAG*) required for glucose metabolism/glycolysis. To understand the relationship between *RAG* and *KIHSL1*, *rag* and *Klhlsl1Δ* mutant strains were investigated. The analysis showed that all the mutants contained a phosphorylated form of Hog1 and displayed an inability to synthesize/accumulate glycerol as a compatible solute. In addition, *rag* mutants also showed alterations in both cell wall and membrane fatty acids. The pleiotropic defects of these strains indicate that a common pathway regulates glucose utilization and stress response mechanisms, suggesting impaired adaptation of the plasma membrane/cell wall during the respiratory–fermentative transition. KIHsl1 could be the link between these adaptive pathways and the morphogenetic checkpoint.

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INTRODUCTION

In *Saccharomyces cerevisiae*, the cell cycle is regulated by the Cdc28 kinase and its regulatory subunits (cyclins). Swe1 kinase negatively regulates entry into mitosis through inhibitory phosphorylation of Cdc28 (Booher *et al.*, 1993). Swe1 accumulates through the late G₁ and S phases, and is then degraded in G₂ by hyperphosphorylation (Sia *et al.*, 1998). *HSL1*, also called *NIK1*, encodes a protein kinase, which is a negative regulator of Swe1 (Tanaka & Nojima, 1996; Ma *et al.*, 1996). Hsl1 is a component of the septin morphogenetic checkpoint that promotes bud development and the recruitment of Swe1 at the septin ring where it is phosphorylated/degraded (McMillan *et al.*, 1999; Lew, 2003).

S. cerevisiae is a Crabtree-positive yeast in that respiration is completely repressed by glucose even under aerobic

conditions, growth being supported solely by fermentation (Gancedo, 1998). In contrast, in the Crabtree-negative yeast *Kluyveromyces lactis* both respiratory and fermentative pathways co-exist during growth on glucose (De Deken, 1966; Breunig *et al.*, 2000), although respiration appears to be dispensable since antimycin A (AA) does not inhibit growth on glucose (Rag^+ phenotype) (Goffrini *et al.*, 1989; Wésolowski-Louvel *et al.*, 1992a). Sensitivity to AA on glucose (Rag^-) is associated with mutations in genes encoding glycolytic enzymes, glucose transporters and sensing components, as well as their regulatory factors (Goffrini *et al.*, 1991; Wésolowski-Louvel *et al.*, 1992b; Prior *et al.*, 1993; Bianchi *et al.*, 1996; Prior *et al.*, 1996; Blaisonneau *et al.*, 1997; Betina *et al.*, 2001; Lemaire & Wésolowski-Louvel, 2004; Hnatova *et al.*, 2008).

During the characterization of the putative cell cycle gene *KIHSL1* in *K. lactis*, we found that its deletion led to a Rag^- phenotype, suggesting involvement in glucose metabolism. To identify the relationship between *RAG* and *KIHSL1*, *rag* and *Klhlsl1Δ* mutant strains were further analysed. All *rag*

Abbreviations: AA, antimycin A; CWI, cell wall integrity; FACS, fluorescence-activated cell sorting; MAPK, mitogen-activated protein kinase.

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mutants showed impaired glycerol accumulation in relation to the activation of HOG. Moreover, these mutants also displayed altered membrane fatty acid content and a cell wall more sensitive to stress agents compared to wild-type.

METHODS

Strains, media and culture conditions. The strains used in this work are listed in Table 1. Double mutant strains were constructed mating the single *rag1* (PM6-12A), *rag6* (MW144-3B) or *rag12* (MW186-3C) point mutants (Wésolowski-Louvel *et al.*, 1992a) with the MW278-20C/*Klhl1Δ* strain. The diploids were selected and sporulated, and the asci were dissected. Tetrad analysis from each diploid allowed the isolation of spores in which AA sensitivity and G418 resistance co-segregated (2 *Rag*⁺ G418^r; 2 *Rag*[−] G418^s). We selected from each cross two double *rag1-1 Klhl1Δ* mutants, two double *rag6-1 Klhl1Δ* mutants and three double *rag12-1 Klhl1Δ* mutants (see Table 1).

Cultures were grown with shaking at 28 °C in YP (1 % Difco yeast extract, 2 % Difco Bacto-peptone) supplemented by carbon sources at

the concentration specified in the text. In order to cause osmotic stress, NaCl was added to a final concentration of 1 M to YP containing 1 % glucose (YPD1) (giving an *a_w* of 0.967, corresponding to a water potential of −4.2 MPa). AA was added at 5 μM in YPD5 plates (Goffrini *et al.*, 1989). The geneticin (G418) concentration in YPD plates was 100 μg ml^{−1}. YPD plates were also supplemented with ergosterol at 20 μg ml^{−1} (stock: 0.2 g Tween 80 in 50 % water, 50 % ethanol).

Deletion of *KIHSL1*. The *KIHSL1* gene was isolated from a *K. lactis* genomic bank constructed in the replacement vector λ L47. The *KIHSL1* locus was subcloned and the nucleotide sequence was determined. The construction of the deletion cassette was performed by cloning a 3.0 kbp *EcoRI*–*Bam*HI fragment and a 3.7 kbp *Bam*HI fragment into the corresponding sites of the pBluescript II SK vector (Stratagene). This plasmid contained the entire gene plus 2 kbp upstream of the ATG sequence and 650 bp of the 3' untranslated sequence. The deletion cassette was constructed by inserting the 1.45 kbp *Eco*CR–*Sma*I *KanMX4* gene conferring G418 resistance into the filled-in *Nco*I sites of *KIHSL1*. We removed 2.6 kbp of the gene, leaving 560 bp after the ATG (first 192 aa) and 620 bp upstream the stop codon. The linearized cassette (after *Hpa*I digestion) was

Table 1. Yeast strains used in this study

Strain	Genotype*	Reference
MW278-20C	<i>MATα ade-T600 uraA1-1 lac4-8</i>	Wésolowski-Louvel collection
MW270-7B	<i>MATα leu2 metA1-1 uraA1-1</i>	Blaisonneau <i>et al.</i> (1997)
MW270-7B/ <i>Δrag1</i>	Isogenic to MW270-7B except <i>rag1::URA3</i>	Billard <i>et al.</i> (1996)
CBS2359/152	<i>MATα metA1-1</i>	Heipieper <i>et al.</i> (2000)
GG1996	<i>MATα rag2::loxP</i>	Steensma and Ter Linde (2001)
PM6-7A	<i>MATα ade-T600 uraA1-1</i>	Wésolowski-Louvel <i>et al.</i> (1992a)
PM6-7A/ <i>Klcox18Δ</i>	Isogenic to PM6-7A except <i>Klcox18::URA3</i>	Hikkel <i>et al.</i> (1997)
PM6-7A/ <i>pdcl1Δ</i>	Isogenic to PM6-7A except <i>Klpc1::URA3</i>	Bianchi <i>et al.</i> (1996)
PM6-7A/VV23	<i>MATα ade-T600 uraA1-1 rag3-1</i>	Prior <i>et al.</i> (1996)
PM6-7A/VV32	<i>MATα ade-T600 uraA1-1 rag4-1</i>	Betina <i>et al.</i> (2001)
PM6-7A/VV18	<i>MATα ade-T600 uraA1-1 rag7-1</i>	Wésolowski-Louvel <i>et al.</i> (1992a)
PM6-7A/VV30	<i>MATα ade-T600 uraA1-1 rag8-1</i>	Blaisonneau <i>et al.</i> (1997)
PM6-7A/VV48	<i>MATα ade-T600 uraA1-1 rag10-1</i>	Wésolowski-Louvel <i>et al.</i> (1992a)
PM6-7A/VV10	<i>MATα ade-T600 uraA1-1 rag11-1</i>	Wésolowski-Louvel <i>et al.</i> (1992a)
PM6-7A/VV12	<i>MATα ade-T600 uraA1-1 rag12-1</i>	Wésolowski-Louvel <i>et al.</i> (1992a)
PM6-7A/VV36	<i>MATα ade-T600 uraA1-1 rag13-1</i>	Neil <i>et al.</i> (2004)
PM6-7A/VV15	<i>MATα ade-T600 uraA1-1 rag14-1</i>	Wésolowski-Louvel <i>et al.</i> (1992a)
PM6-12A	<i>MATα ade-T600 uraA1-1 rag1-1</i>	Wésolowski-Louvel collection
MW144-3B	<i>MATα lysA1 uraA1-1 rag6-1</i>	Wésolowski-Louvel collection
MW186-3C	<i>MATα ade-T600 lysA1 trpA1 rag12-1</i>	Wésolowski-Louvel collection
MW278-20C/ <i>Klhl1Δ</i>	Isogenic to MW278-20C except <i>Klhl1::kanMX4</i>	This work
MWLK1136	<i>adeT-600 leu2 lysA1 trpA1 rag12-1 Klhl1::kanMX4</i>	This work
MWLK1137	<i>adeT-600 leu2 uraA1 rag12-1 Klhl1::kanMX4</i>	This work
MWLK1138	<i>adeT-600 rag12-1 Klhl1::kanMX4</i>	This work
MWLK1140	<i>adeT-600 uraA1 rag1-1 Klhl1::kanMX4</i>	This work
MWLK1141	<i>adeT-600 uraA1 rag1-1 Klhl1::kanMX4</i>	This work
MWLK1142	<i>lysA1 uraA1 rag6-1 Klhl1::kanMX4</i>	This work
MWLK1143	<i>adeT-600 leu2 uraA1 rag6-1 Klhl1::kanMX4</i>	This work

**RAG* genes encode a low-affinity glucose transporter (*RAG1*, Wésolowski-Louvel *et al.*, 1992b), phosphoglucose isomerase (*RAG2*, Goffrini *et al.*, 1991), a regulator of pyruvate decarboxylase (*RAG3*, Prior *et al.*, 1996), a glucose sensor (*RAG4*, Betina *et al.*, 2001), a pyruvate decarboxylase (*RAG6/KIPDC1*, Bianchi *et al.*, 1996), casein kinase 1 (*RAG8*, Blaisonneau *et al.*, 1997), DL-glycerol-3-phosphatase (*RAG12*, unpublished), a transcriptional activator of glycolytic genes (*RAG13/KIGCR1*, Neil *et al.*, 2004) and the β subunit of phosphofructokinase (*RAG14*, Wésolowski-Louvel *et al.*, 1992a). The functions of *RAG7*, *RAG10*, *RAG11* are unknown.

transformed into strain MW278-20C for gene replacement. Positive clones selected on G418 plates were analysed for the integration of the cassette into the *KIHSL1* locus first by PCR and then by Southern analysis. The genomic DNA of G418-resistant and wild-type strains was digested with *Bam*HI and *Hpa*I. Restriction fragments, separated by gel electrophoresis, were transferred onto a nylon filter and probed with the *Pml*I–*Xba*I 1.6 kbp *KIHSL1* fragment labelled with 32 P. Autoradiography showed two signals of 0.8 and 1 kbp in the wild-type, as expected, and two bands of 0.82 and 2.7 kbp in the deletants, confirming the correct integration of the cassette into the *KIHSL1* locus.

Complementation tests were performed with the KCplac13 centromeric plasmid containing the *KIHSL1* gene. This plasmid was constructed in two steps. First, we cloned the 1.9 kbp *Sca*I–*Bam*HI fragment (promoter plus 5' end of the gene) into the *Eco*ICR–*Bam*HI site of the plasmid. Then we cloned the remaining part of the gene and terminator as a 3.7 kbp *Bam*HI fragment into the selected plasmid.

Assay methods. Glycerol concentration in culture supernatants and in cell extracts was assayed using a commercial kit from R-Biopharma, according to the manufacturer's instructions. Intracellular glycerol was determined from cell extracts obtained from 5 ml culture aliquots grown to an OD₆₀₀ of 2.0, resuspended in 0.5 ml 0.5 M Tris, pH 7.5, and treated as described by Albertyn *et al.* (1994).

Electron microscopy. Cells grown on YPD until late-exponential phase were fixed with 2 % glutaraldehyde in distilled water for 1 h at room temperature and washed with water. To reveal cellular membranes without removing the cell wall, cells were post-fixed and prepared as described previously (Uccelletti *et al.*, 2000). Ultrathin sections were stained with lead citrate before examination with a transmission electron microscope at 80 kV.

Flow cytometry analysis. Fluorescence-activated cell sorting (FACS) analysis was performed on cells grown to late-exponential phase, as described previously (Uccelletti *et al.*, 1999).

Lipid extraction and transesterification. Flasks containing 100 ml YPD medium with or without stress agents were inoculated with *rag*, *Klhlsl1Δ*, *Klcox18Δ* and wild-type strains at a concentration of 1×10^5 – 2×10^5 cells ml⁻¹. When the cultures reached a titre of 0.9–1.0 (OD₆₀₀), cells were harvested, washed with phosphate buffer (50 mM, pH 7.0) and the lipids were extracted with chloroform/methanol/water as described by Bligh & Dyer (1959). Fatty acid composition was determined as described previously (Heipieper *et al.*, 2000).

Protein extracts and immunoblot analysis. *K. lactis* strains were grown with shaking at 28 °C in 20 ml 1 % YPD1, with or without 1 M NaCl to and OD₆₀₀ of 0.9–1.0. Cells were harvested in NP-40 Cell Lysis Buffer (Invitrogen) and lysed by vigorous shaking with glass beads (0.45 mm diam.). Cell extracts were separated from cell debris by centrifugation at 13 000 r.p.m. for 15 min at 4 °C. Equal amounts of proteins (60 µg) from each strain were boiled for 5 min, run onto a 8 % polyacrylamide gels and transferred to PVDF membranes (Bio-Rad). Blots were probed overnight at 4 °C with an mAb to phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180-Tyr182) (Cell Signaling Technology) at 1:1000 dilution, or with polyclonal antibody to *S. cerevisiae* Hog1 (Santa Cruz Biotechnology) at 1:500 dilution in the presence of 5 % non-fat milk. The primary antibody was detected using an HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) using the ECL detection system (Perkin Elmer).

Zymolyase assay. Cells were grown in YPD with or without 1 M NaCl or AA to 0.9×10^8 – 1×10^8 cells ml⁻¹. Cells (5×10^8) were

collected, resuspended in 4 ml sorbitol buffer (20 mM Tris containing 1.2 M sorbitol and 10 mM MgCl₂, pH 7.2) and treated for 10 min with 3 % 2-mercaptoethanol. Then, 12.5 U zymolyase 100T (Seikagaku Kogyo) was added and incubated at 30 °C with gentle agitation. Spheroplast lysis was determined, after dilution in water, at time intervals by measuring OD₆₆₀.

RESULTS

A *KIHSL1* deletion mutant shows altered morphology and DNA content

The gene that encodes a putative serine–threonine kinase in *K. lactis* has been identified (EMBL accession no. AJ555232). This protein belongs to one of the largest and most conserved enzyme families catalysing phosphotransfer reactions (Rubenstein & Schmidt, 2007). Comparison of the *K. lactis* protein with known databases showed the highest similarity score with the *S. cerevisiae* Hsl1/Nik1 kinase (Tanaka & Nojima, 1996; Ma *et al.*, 1996). Besides the almost identical kinase domain and the different length of the two proteins (Hsl1, 1518 aa; KlHsl1, 1267 aa), the alignment was conserved along the entire sequence.

To characterize this gene, we constructed a disruption module to replace the coding region with the *kanMX4* gene and introduced it into strain MW278-20C. Transformants, selected for their ability to grow on G418, were analysed by PCR and Southern blotting to confirm the integration of the cassette into the *KIHSL1* locus. One of these strains, MW278-20C/*Klhlsl1Δ*, was selected and used for further analysis.

The *Klhlsl1Δ* mutant was studied at the morphological level and by FACS analysis. Electron microscopic analysis showed mutant cells (Fig. 1b, c) with altered morphology, elongated buds with a cytokinesis defect and a cell wall that was at least twice as thick as that of the wild-type strain (Fig. 1a). In *S. cerevisiae*, the elongated bud morphology is typical of septin mutants in G₂ progression (Burton & Solomon, 2000), resulting at least in part from a cell cycle delay imposed by the Cdc28-inhibitory kinase Swe1. For this reason we looked at the DNA content of the wild-type and mutant strains by FACS. As reported in Fig. 1(d), the DNA content of *KIHSL1* cells showed two peaks, one corresponding to cells with a pre-replication DNA content (*n*), the other corresponding to cells with a post-replication DNA content (*2n*) (Uccelletti *et al.*, 1999). On the contrary, in *Klhlsl1Δ*, a greater proportion of cells had DNA content greater than *2n*, supporting a role for KlHsl1 in the cell cycle of *K. lactis*.

The Rag[−] phenotype is associated with sensitivity to osmotic stress

The *Klhlsl1Δ* mutant showed slightly reduced growth in almost all conditions tested when compared to its parent strain. However, growth was further reduced when cultures were grown on YPD plates containing NaCl or in the presence of the respiratory inhibitor AA (Fig. 2).

Fig. 1. Electron micrographs of wild-type (a) and *Klhsl1Δ* cells (b, c). The insets show details of the cell wall at higher magnification. cw, Cell wall; er, endoplasmic reticulum; G, Golgi body; m, mitochondria; n, nucleus; v, vacuole. Bars, 2 μ m. (d) FACS analysis of MW278-20C (WT) and *Klhsl1Δ* (Δ) cells grown on YPD until late-exponential phase.

In *K. lactis*, sensitivity to AA (*Rag*[−] phenotype) allows the isolation of genes encoding glycolytic enzymes and for glucose utilization activities (see Table 1). To test whether *Klhsl1Δ* and *rag* mutants might also share sensitivity to

Fig. 2. Growth tests of wild-type, *rag* and *Klhsl1Δ* mutants. Cells were grown on YPD plates with or without AA (YPD+AA) or NaCl (YPD+NaCl). Cultures were adjusted to the same density and 5 μ l of serial 10-fold dilutions were spotted onto the indicated medium. The initial concentration was 1×10^7 cells ml^{−1}. Strains: MW278-20C (WT), *Klhsl1Δ* (Δ), *Klhsl1Δ* transformed with *KIHL1* (Δ +HSL1), PM6-7A (WT₁), GG1996 (*rag2*), PM6-7A/VV23 (*rag3*), PM6-7A/VV30 (*rag4*), PM6-7A/VV18 (*rag7*), PM6-7A/VV48 (*rag10*), PM6-7A/VV10 (*rag11*), PM6-7A/VV36 (*rag13*), PM6-7A/VV15 (*rag14*).

NaCl, these strains were grown on YPD plates containing 1 M NaCl and, as a control, on AA. Indeed, as in the *Klhsl1Δ* mutants, the two phenotypes seems to be associated. In fact, all the *rag* mutants were also sensitive, although at different extent, to NaCl (Fig. 2). The ability to grow in the presence of high concentrations of NaCl causes osmotic stress that cells counteract by intracellular accumulation of glycerol (Blomberg & Adler, 1992; Hohmann, 2002). Therefore, we determined the content of glycerol in both the supernatants and the cell extracts of wild-type strains and *rag* mutants grown in glucose medium with or without osmotic stress or AA. As shown in Table 2, wild-type strains grown on YPD accumulated extracellular glycerol (0.44–0.8 g l^{−1}), while in the presence of AA the accumulation of glycerol was higher (1.3 g l^{−1}), albeit with different kinetics. Strain MW278-20C accumulated a slight amount of glycerol inside the cells as well in the presence of AA, compared to strain PM6-7A, confirming the different behaviour of the two wild-type strains. In *S. cerevisiae*, glycerol accumulates in the supernatant as a redox-balancing sink during anaerobiosis (Björkqvist *et al.*, 1997), and in *K. lactis* glycerol accumulation can be attributed to the block of the respiratory chain by AA. In contrast to AA cultures, wild-type strains growing in the presence of NaCl had no glycerol in the supernatant. Under these conditions, as shown in Table 2, to counteract the extracellular osmotic pressure glycerol accumulates inside the cell at levels tenfold higher than those of the wild-type strains grown in YPD. In *S. cerevisiae*, osmostressed cells accumulated high intracellular amounts of glycerol, reflecting increased synthesis, retention and accumulation. The plasma membrane aquaglyceroporin Fps1, a protein also conserved in *K. lactis* (Tamás *et al.*, 2003), prevents glycerol diffusion by turgor-mediated closure of its channel (Tamás *et al.*, 1999; Hohmann, 2002). Unexpectedly, *Klhsl1Δ* and *rag* mutants were unable to accumulate intra- and extracellular glycerol at levels comparable to those of the

Table 2. Glycerol determination in supernatants and cell extracts of wild-type and *rag* mutant strains

Glycerol concentration (g l^{-1}) was determined in the supernatants of wild-type and mutant strain cultures at increasing cell densities (OD_{600}) from cultures grown on YPD1, YPD1 + 1 M NaCl (NaCl) or YPD5 + AA (AA). Intracellular glycerol values are reported in bold type and were determined from cell extracts obtained from 5 ml cultures grown to an OD_{600} of 2.0. The glycerol content in YP medium was $0.12 \pm 0.01 \text{ g l}^{-1}$. Each value is the mean from 3–5 independent determinations.

Strain	OD_{600}			
	1.0	2.0	3.0	5.0
MW278-20C (NaCl)	0.12 ± 0.01	0.13 ± 0.02 (0.01 ± 0.05)	0.04	0.03
MW278-20C (AA)	1.08 ± 0.04	1.26 ± 0.02 (0.30 ± 0.03)	1.31 ± 0.03	1.32 ± 0.025
MW278-20C	0.18 ± 0.01	0.30 ± 0.02 (0.11 ± 0.02)	0.54 ± 0.02	0.80 ± 0.035
MW278-20C/ <i>Klhl1</i> Δ	0.11 ± 0.01	0.13 ± 0.01 (0.15 ± 0.02)	0.23 ± 0.01	0.35 ± 0.02
PM6-7A (NaCl)	0.07	0.03 (0.64 ± 0.04)	<0.03	<0.03
PM6-7A (AA)	0.49 ± 0.02	0.75 ± 0.03 (0.12 ± 0.01)	1.15 ± 0.04	1.30 ± 0.035
PM6-7A	0.13 ± 0.01	0.22 ± 0.02 (0.04 ± 0.01)	0.28 ± 0.02	0.44 ± 0.03
PM6-7A/VV23 (<i>rag</i> 3)	0.12 ± 0.01	0.13 ± 0.01 (0.03)	0.12 ± 0.01	0.10 ± 0.01
PM6-7A/VV32 (<i>rag</i> 4)	0.13 ± 0.01	0.11 ± 0.01 (0.04 ± 0.01)	0.10 ± 0.01	0.10 ± 0.01
PM6-7A/ <i>pdcl1</i> Δ (<i>rag</i> 6)	0.12 ± 0.01	0.11 ± 0.01 (0.03)	0.10 ± 0.01	0.09 ± 0.01
PM6-7A/VV30 (<i>rag</i> 8)	0.07	0.07 (0.04 ± 0.01)	0.08 ± 0.01	0.10 ± 0.01
PM6-7A/VV12 (<i>rag</i> 12)	0.12 ± 0.01	0.13 ± 0.01 (0.03)	0.15 ± 0.01	0.40 ± 0.02
PM6-7A/VV36 (<i>rag</i> 13)	0.13 ± 0.01	0.14 ± 0.01 (0.06 ± 0.01)	0.14 ± 0.02	0.08
PM6-7A/VV36 (<i>rag</i> 13)	0.13 ± 0.01	0.14 ± 0.01 (0.06 ± 0.01)	0.14 ± 0.02	0.08

wild-type strains, nor were they able to transport it inside even in YPD (Table 2), although we cannot rule out the possibility that these mutants could metabolize glycerol.

Klhl1 Δ and *rag* mutants display the phosphorylated form of KlHog1

The sensitivity of the *rag* mutants to NaCl is probably caused by an inability to accumulate intracellular glycerol during growth on glucose. In *S. cerevisiae*, this condition, associated with mutations in glycerol accumulation pathways, activates the high-osmolarity glycerol (HOG) pathway by longer periods of Hog1 phosphorylation (Klipp *et al.*, 2005; Hohmann, 2009). Therefore, we compared the activation of the mitogen-activated protein kinase (MAPK) KlHog1 by phosphorylation (pHog1) in wild-type and *Klhl1* Δ strains in YPD, and in response to the addition of NaCl. As expected (Fig. 3), pHog1 was absent in the wild-type strain grown in YPD, but it was activated slightly in NaCl-containing medium. On the contrary, in both *Klhl1* Δ and *rag* mutants, pHog1 was present, although at different levels, even in YPD, confirming the presence of severe stress conditions in these strains. The diverse amounts of pHog1 in *rag* mutants might reflect the different lengths of time of adaptation to stress conditions which, as in *S. cerevisiae* (Hohmann, 2009), could be specific for each mutant.

rag mutants display plasma membrane and cell wall alterations

The composition and fluidity of the yeast plasma membrane vary according to fermentative potential due

to the toxicity of ethanol for yeast cells that is counteracted by modifications in membrane composition (Casey & Ingledew, 1986; Jones & Greenfield, 1987). Since RAG genes encode glycolytic/glucose-metabolizing activities, we wondered if there are any differences in the membrane composition of *rag* mutants. Therefore, the fatty acid content of wild-type and *rag* mutants grown in YPD medium with and without stress compounds was analysed (Heipieper *et al.*, 2000). As a control, we also determined the fatty acid content of the respiratory-deficient osmo-sensitive strain *Klcox18* Δ (Table 1; Hikkel *et al.*, 1997; Goffrini, 2007). The composition of the membrane of the wild-type grown in YPD was shown to be very different from that obtained from YPD containing NaCl or AA (Fig. 4a). Strikingly, the fatty acid pattern of the wild-type grown under stress conditions was similar to that of the *rag* and

Fig. 3. Hog1 activation in *Klhl1* Δ and *rag* mutants. Strains were grown in YPD with or without NaCl. Cells were lysed and the proteins were separated and transferred to membranes. Blots were probed with antibody to phospho-p38 MAP kinase (Thr180-Tyr182), stripped and reprobed with *S. cerevisiae* Hog1. Strains: MW278-20C (WT), PM6-7A (WT₁), *Klhl1* Δ (Δ), MW270-7B/ Δ *rag1* (*rag*1), GG1996 (*rag*2), PM6-7A/VV23 (*rag*3), PM6-7A/VV30 (*rag*4), PM6-7A/*pdcl1* Δ (*rag*6), PM6-7A/VV30 (*rag*8), PM6-7A/VV10 (*rag*11).

Fig. 4. (a) Determination of fatty acids in wild-type and *rag* mutants. Fatty acid content was measured in strains PM6-7A (WT, WT+AA and WT+NaCl), PM6-7A/*Klcox18*Δ (*Δcox18*) and *rag* mutants r4 (*rag4*), r6 (*rag6*), r8 (*rag8*), r12 (*rag12*) and r13 (*rag13*) from cultures grown on YPD with or without AA or 1 M NaCl. Results are means ± SEM of two–three independent experiments. (b, c) *K. lactis* cell lysis sensitivity of wild-type and mutant strains following treatment with Zymolyase. Cells were grown in YPD with or without NaCl or AA, harvested and treated with the enzyme. Cell lysis was measured at time intervals by the decrease in OD₆₆₀. Results are means ± SEM of three independent experiments. (b) ■, WT (PM6-7A); △, WT+NaCl; □, WT+AA; ●, r6; ▲, r8. (c) ■, WT (MW278-20C); △, WT+NaCl; □, WT+AA; ◆, *Klhsl1*Δ.

respiratory-deficient *Klcox18*Δ mutants grown in YPD. In particular, the wild-type grown in YPD showed a drastic decrease (7–10 %) in oleic acid (C18:1Δ9*cis*) and a great increase (15–20 %) in polyunsaturated linolenic acid (C18:3Δ9,12,15*cis,cis,cis*) (indicated in Fig. 4a with *), in addition to a slightly reduced content (2–6 %) of palmitic, palmitoleic and linoleic acids (C16:0, C16:1Δ9*cis*, C18:2Δ9,12*cis,cis*), compared to *Klcox18*Δ and *rag* mutants.

The almost identical fatty acid content of the *rag* mutants and the wild-type grown in NaCl/AA-containing medium suggested that a ‘compensatory mechanism’ (Popolo *et al.*, 2001) might also be implicated in the cell wall integrity (CWI) pathway. This stress response mechanism is triggered by changes in the proportion of cell wall polymers, and this pathway may be inefficient in *rag* mutants as suggested by the increased sensitivity of the phosphoglucose isomerase mutant (*rag2*) to cell-wall-degrading zymolyase activities (Overkamp *et al.*, 2002). Moreover, in *S. cerevisiae*, it has been shown that the HOG and the Slt2 pathways activate the CWI pathway in response to zymolyase (Bermejo *et al.*, 2008). Since these signalling cascades also seem to be

conserved in *K. lactis* (Kirchrath *et al.*, 2000; Rodicio *et al.*, 2008), and as we have reported the activation of KlHog1 (Fig. 3), we asked whether the increased sensitivity to zymolyase could be extended to other *rag* mutants in comparison to the wild-type. Indeed, as shown in Fig. 4(b, c), the sensitivity to zymolyase shown by *rag6* (pyruvate decarboxylase mutant), *rag8* (casein kinase mutant) and *Klhsl1*Δ mutants was comparable to that of the wild-type strains grown in NaCl/AA-containing medium, indicating impaired CWI response mechanisms. Although these results do not necessarily mean that *rag* mutants display a thicker cell wall, like *Klhsl1*Δ (Fig. 1b, c), they certainly have a different cell wall polymer composition compared to the wild-type strains.

Addition of ergosterol recovers the Rag[−] phenotype

The fatty acid composition of *Klhsl1*Δ and *rag* mutants also suggested defects in the biosynthesis of membrane components, such as fatty acids and ergosterol, a major structural component required for the control of

membrane fluidity (Parks *et al.*, 1995). To test this hypothesis, *Klhl1Δ* and *rag1* (MW270-7B/ Δ *rag1*), *rag6* (PM6-7A/*pdcl1Δ*) and *rag8* (PM6-7A/VV30) (see Table 1) mutants with the most severe Rag[−] phenotype, were grown in YPD plates containing ergosterol to see if it could recover the growth inhibition caused by AA and/or osmotic stress. Indeed, as shown in Fig. 5, ergosterol not only helped to recover the growth of the four mutants on AA, but it also had a positive growth effect on both wild-type strains and on the other *rag* mutants (not shown). However, this effect was limited to growth with AA because ergosterol was unable to relieve the sensitivity to NaCl (not shown). The addition of neither fatty acids nor *myo*-inositol was capable of suppressing the growth defect of the mutants (not shown).

Growth analysis of *rag Klhl1Δ* double mutants

Double *rag Klhl1Δ* mutants were obtained by crossing the *rag1* (PM6-12A strain), *rag6* (MW144-3B strain) and *rag12* (MW186-3C strain) mutants (see Table 1), carrying a point mutation, with the *Klhl1Δ* mutant (see Methods). These strains were tested in an attempt to search for genetic interactions amongst the *KIHSL1* and *RAG* genes by growth on YPD plates containing AA, NaCl or H₂O₂. The plates supplemented with H₂O₂ were included in the analysis to compare the sensitivity of the double versus the single mutants to oxidative stress. Interestingly, all the double mutants showed slightly reduced growth in YPD containing NaCl compared to their single mutant counterparts (Fig. 6). Moreover, all the double mutants, with the exception of the *rag6 Klhl1Δ* mutant, showed reduced growth on both YPD alone and with H₂O₂. It must be emphasized that the double mutants, like the single mutants, recovered growth in the presence of AA when ergosterol was added to the plates (not shown), although they were unable to accumulate both intra- and extra-cellular glycerol.

Fig. 5. Growth tests of wild-type, *rag* and *Klhl1Δ* mutants on ergosterol plates. Cells were grown on YPD plates with or without NaCl, AA and AA plus ergosterol. Cell dilutions are as described in the legend to Fig. 2. Strains: PM6-7A (WT₁), MW270-7B (WT₂), MW278-20C (WT), *Klhl1Δ* (*hsl1*), *rag1* (MW270-7B/ Δ *rag1*), *rag6* (PM6-7A/*pdcl1Δ*), *rag8* (PM6-7A/VV30).

Fig. 6. Growth tests of wild-type, *rag* and *Klhl1Δ* mutants and *rag-Klhl1Δ* double mutants. Cells were grown on YPD plates with or without AA, NaCl and H₂O₂. Cell dilutions are as described in the legend to Fig. 2. Strains: PM6-7A (WT₁), MW278-20C (WT₂), *rag1* (r1), *rag6* (r6), *rag12* (r12), *Klhl1Δ* (Δ), MWLK1140 / MWLK1141 (*rag1 Klhl1Δ*=r1/ Δ), MWLK1142/ MWLK1143 (*rag6 Klhl1Δ*=r6/ Δ), MWLK1136/ MWLK1137/MWLK1138 (*rag12 Klhl1Δ*=r12/ Δ).

DISCUSSION

The properties of *KIHSL1* were studied to understand the function of this gene, which is involved in cell cycle control and morphogenesis, in Crabtree-negative and -positive yeasts. The observation that *Klhl1Δ* displayed the Rag[−] phenotype led to a further investigation to search for the metabolic relationship between *Klhl1Δ* and *rag* mutants.

Nineteen complementation groups have been characterized to date by the genetic dissection of the Rag[−] phenotype. Complementation of these mutants has allowed the isolation of genes encoding activities involved in the utilization of glucose and glycolytic enzymes (see Table 1). In this study, we report that *Klhl1Δ* and *rag* mutants displayed enhanced sensitivity to osmotic stress conditions caused by their inability to accumulate/produce glycerol. Yeast cells produce and accumulate glycerol from an intermediate of glycolysis, dihydroxyacetonephosphate, as the main compatible solute for redox balancing and osmoregulation (Ansell *et al.*, 1997; Serrano *et al.*, 1997; Hohmann, 2009). Therefore, the impaired glycolytic flux of the *rag* mutants (Goffrini *et al.*, 1989; Lemaire & Wésolowski-Louvel, 2004) is unable to support the extracellular accumulation of glycerol necessary for cytoplasmic reoxidation of excess NAD(P)H (van Dijken & Scheffers, 1986). As a consequence, the metabolism of these cells becomes respirofermentative (González-Siso *et al.*, 2000) and the respiratory chain intervenes to neutralize the excess NAD(P)H (Overkamp *et al.*, 2002). It follows that

the addition of AA blocks the respiratory chain, inhibiting the growth of these mutants. Moreover, since Hog1 controls many of the steps leading to the intracellular accumulation of glycerol, the primary mechanism of resistance to high salt (Hohmann, 2002), *rag* mutants behave like *S. cerevisiae* glycerol-production mutants (Klipp *et al.*, 2005), displaying the phosphorylated form of this MAPK.

The fatty acid content, in particular the oleic and linolenic acid levels of wild-type cells grown under stress (Fig. 4a) or with increasing concentrations of ethanol (Heipieper *et al.*, 2000), was shown to be very similar to the *rag* mutant pattern, but different to the wild-type counterpart grown in YPD. Finally, *rag* mutants are more sensitive to zymolyase, similar to wild-type strains grown under stress, a condition that in *S. cerevisiae* has been associated with CWI defects (Bermejo *et al.*, 2008). All these data, inferred from *rag* and wild-type cells, indicated that common adaptive pathways are responsible for plasma membrane and cell wall dynamics activated under stress: (a) pathways that block the fluctuation of the membrane fatty acids/cell wall polymers; and (b) metabolic routes that lead to the intra-/extracellular accumulation of glycerol. The altered dynamics of the membranes were further confirmed by the ability of mutants to recover growth on AA after the addition of ergosterol, a major structural component required for fluidity, permeability and/or strengthening of the phospholipid membrane bilayer. The expression in *S. cerevisiae* of two *K. lactis* desaturase genes (*KIFAD2* and *KIFAD3*), which are not present in *S. cerevisiae* and which encode activities that convert oleic (18:1) into linoleic (18:2) and linolenic acids (18:3), has been reported and these genes confer adaptation to alkaline growth conditions (Yazawa *et al.*, 2009). It will be interesting to test whether *KIFAD2* and/or *KIFAD3* are required for membrane adaptation during the respiratory–fermentative growth transition.

Although we have not demonstrated that *KIHSL1* is the orthologue of *HSL1*, the morphogenetic defects and the altered DNA content of the *Klhlsl1Δ* mutant suggested a block in G₂ (Fig. 1b, c, d). In *S. cerevisiae*, it has been reported that Hsl1 phosphorylates the glycolytic enzyme Gpm1 *in vivo* (Ptacek *et al.*, 2005), while Hog1 phosphorylates a residue, also conserved in *K. lactis*, within the Hsl7 docking site of Hsl1, leading to G₂ delay (Clotet *et al.*, 2006). These data also suggest a link between morphogenetic functions and glycolysis, despite the fact that the *HSL1* deletion mutant showed no phenotype except a morphogenetic defect. The diverse habitat of this Crabtree-positive yeast could account for its greater fermentative capability and adaptation to stress conditions. If the role of *KIHSL1* is conserved, we suggest that KlHsl1 controls, via phosphorylation, an activity in the second part of glycolysis, like Gpm1 in *S. cerevisiae*, leading to glycerol accumulation. Therefore, KlHsl1, as a component of the glycerol/stress response pathways, could link morphogenesis with respiratory–fermentative adaptation.

In agreement with the concordant *rag* and *Klhlsl1Δ* phenotypes reported in this study, the analysis of the double mutants showed a slight growth reduction compared to the single *rag* mutant counterpart. Given that the effects are very limited, this can be considered as a strong indication for epistasis, suggesting that Rag proteins work in the same pathway as KlHsl1, exerting an additional control on stress response mechanisms. However, an extensive genetic analysis will be required to exclude the possibility that the limited growth reduction observed in the double mutants is due to point mutations present in the parental *rag* strains.

It will be interesting to test whether, as in *rag* mutants, stress conditions could block membrane fluctuation in other yeasts/higher eukaryotes with glycerol homeostasis supporting a critical role in adaptation/cell differentiation (Prentki & Madiraju, 2008; Diaz-Ruiz *et al.*, 2009).

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