In the yeast Kluyveromyces lactis, glucose 6-phosphate dehydrogenase (G6PDH) is detected as two differently migrating forms on native polyacrylamide gels. The pivotal metabolic role of G6PDH in K. lactis led us to investigate the mechanism controlling the two activities in respiratory and fermentative mutant strains. An extensive analysis of these mutants showed that the NADP⁺(H)/NADPH(−H)-dependent cytosolic alcohol (ADH) and aldehyde (ALD) dehydrogenase balance affects the expression of the G6PDH activity pattern. Under fermentative/ethanol growth conditions, the concomitant activation of ADH and ALD activities led to cytosolic accumulation of NADPH, triggering an alteration in the oligomeric state of the G6PDH caused by displacement/release of the structural NADP⁺ bound to each subunit of the enzyme. The new oligomeric G6PDH form with faster-migrating properties increases as a consequence of intracellular reduct ox/redox imbalance/NADPH accumulation, which inhibits G6PDH activity in vivo. The appearance of a new G6PDH-specific activity band, following incubation of Saccharomyces cerevisiae and human cellular extracts with NADP⁺, also suggests that a regulatory mechanism of this activity through NADPH accumulation is highly conserved among eukaryotes.

The pentose phosphate pathway (PPP) constitutes the major source of NADPH required for the neutralization of reactive oxygen species, for reductive biosynthetic reactions, and for the production of metabolic intermediates. The glucose 6-phosphate dehydrogenase (G6PDH) activity, a protein highly conserved through evolution (2, 26), catalyzes the rate-limiting NADPH-producing step of this metabolic pathway (18).

Recently, we characterized KlZWF1, the Kluyveromyces lactis gene coding for G6PDH, and showed that this enzymatic activity is required during growth on both respiratory and fermentative carbon sources (33). We developed an assay to detect on native polyacrylamide gels the G6PDH activity in cell extracts. By means of this assay, we detected the presence of a single G6PDH band of activity in extracts prepared from glucose, glycerol, lactate, and acetate cultures, whereas in extracts from ethanol-grown cells, a faster-migrating (lower) band was also detected. The latter band was also present when ethanol was added to cultures growing in glucose, glycerol, lactate, and acetate cultures, whereas in extracts from ethanol-grown cells, a faster-migrating (lower) band was also detected. The latter band was also present when ethanol was added to cultures growing in glucose, glycerol, lactate, and acetate cultures, indicating a dominant effect of this substrate over the others. The expression of the K. lactis gene in Saccharomyces cerevisiae showed the presence of five different migrating G6PDH bands of activity, suggesting a tetrameric organization of the enzyme (33). No duplication of the KlZWF1 gene is present in the K. lactis genome, and a single mRNA transcript is expressed in wild-type cells grown in all carbon sources (33). Finally, both bands of activity disappear in Klzwf1Δ mutants grown in ethanol (33). These data clearly indicate that both activity bands on the gel are from the KlZWF1 gene, the lower band probably originating from the upper one following changes in the oligomeric assembly. The finding that K. lactis cell extracts from ethanol-grown cultures produce two bands of G6PDH on native polyacrylamide gels raised the question whether these activities correspond to the dimeric and tetrameric forms observed for the human enzyme (3, 41).

Since G6PDH plays a key role in the maintenance of the NADP⁺/NADPH redox balance and is required for the optimal growth of K. lactis in the presence of any carbon source (33), we compared the relative abundance of the two G6PDH activity bands in different laboratory strains and mutants that were altered in either respiratory or fermentative metabolism. Herein we show that the two bands of G6PDH represent, as in human activity, different oligomeric states of the enzyme that are probably determined by an in vivo mechanism of inhibition of the G6PDH activity caused by cytosolic accumulation of NADPH.

MATERIALS AND METHODS

Strains, media, and culture conditions. The K. lactis strains used in this work are reported in Table 1. Media preparations and cultures conditions were as previously described (33). Hydrogen peroxide or acetaldehyde was added to yeast extract-peptone-dextrose (YPD) medium at the indicated concentrations.

Gene amplifications and construction of chimeric KlZWF1GFP plasmids. The entire KlZWF1 gene, excised from pTZ19/KlZWF1 (33) as a HindIII/XbaI fragment, was cloned into the multicopy pKL plasmid to harbor pKL-KlZWF1. pKL is a Geneticin resistance pKD1-derived stable multicopy vector (31). This plasmid was also used for cloning and overexpression of genes amplified by PCR from the K. lactis genome. The primers used for the amplification of KIALD4, KIALD6, and KIYPD1 and for the construction of the chimeric KlZWF1GFP genes are reported in Table 1, while those for KIGUT2, KINDE1, and KINDEII have been reported elsewhere (34, 35). KlZWF1GFP was constructed by amplifying the 5’ portion (980 bp of the promoter plus the entire KlZWF1 open reading frame [ORF] without the stop codon) and the 3’ portion (stop codon and
460 bp of the 3′-untranslated sequence) of KlZWF1 from pTZ19/KlZWF1. The amplified PCR blunt-ended fragments were cloned in frame in the HincII site of pTZ18. The selected plasmid containing the entire KlZWF1 gene was digested with EcoRV, a unique site located before the stop codon, and ligated with the EcoICR fragment containing the green fluorescent protein (GFP) gene (35). The final chimeric gene was purified as described (31).

**K. lactis strains**

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<td>CBS collection</td>
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**Target gene and primer**

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</tbody>
</table>

<sup>a</sup> These strains had identical G6PDH patterns.

<sup>b</sup> In the text, this strain is also referred to as adh°.

640 bp of the 3′-untranslated sequence) of KlZWF1 from pTZ19/KlZWF1. The amplified PCR blunt-ended fragments were cloned in frame in the HincII site of pTZ18. The selected plasmid containing the entire gene was digested with EcoRIV, a unique site located before the stop codon, and ligated with the EcoICR fragment containing the green fluorescent protein (GFP) gene (35). The final chimeric gene was purified as an XbaI 3.8-kb fragment and cloned in the KCplac13 centromeric (Kcp-KIZWF1GFP) and multicopy pKL-KlZWF1GFP plasmids (35). Yeast transformation and total RNA extraction were performed as previously described (31).

**G6PDH native assay.** *K. lactis* cells extracts, native polycarylamide gels, electrophoresis conditions, and G6PDH staining assays were carried out as previously described (33). For the G6PDH *in vitro* assay, the protein concentration was determined (8), and 10 μg of total protein extract in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.15% Triton X-100 containing protease inhibitor cocktail (Complete; Roche) were incubated overnight on ice with 1 to 15 mM NADP<sup>+</sup> (H) (N0050 and N1630; 150 mM stock in 0.1 M Tris-HCl [pH 8.0]; Sigma) in a final volume of 10 μl. The extracts were then run on native gels and stained as previously described (33). G6PDH activity was assayed by measuring with a spectrophotometer the rate of NADP<sup>+</sup> reduction at 340 nm in TE buffer (0.1 M Tris-HCl [pH 8], 1 mM EDTA) containing NADP<sup>+</sup> at the indicated concentrations.

**Human embryonal (RD) and alveolar (RH30 and RH4) rhabdomyosarcoma (RMS) cell lines,** generously provided by C. Dominici (Sapienza University of Rome), were maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 10 μg/ml streptomycin (Euroclone) in the presence of 10% heat-inactivated fetal bovine serum (Gibco) in a humidified atmosphere with 5% CO<sub>2</sub>, at 37°C. Total protein extracts were prepared from harvested cells, washed with phosphate-buffered saline, lysed with a 20 mM Tris-HCl (pH 7.2) buffer containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 250 μM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete; Roche) for 30 min on ice. Equal amounts of total protein extract (30 μg), incubated overnight on ice with NADP<sup>+</sup> (H) as was done for *K. lactis* extracts, were loaded for each lane, separated on a native polyacrylamide gel, and stained for G6PDH activity.

**Determination of NAD(P)H content.** Harvested cells from 40 ml of YP-glycerol-grown or ethanol-grown cultures were resuspended in 400 μl ofTE containing inhibitors. Cells were broken with glass beads on a vortex apparatus for 10 min, and the supernatants were recovered by centrifugation (33). The protein content of the extracts was determined (8) following treatment with 1% streptomycin sulfate to remove nucleic acids. The low-molecular-mass material was removed from the proteins extracts by...
using ultrafiltration devices with a cut-off of 10 kDa (Vivaspin; Sartorius). The amount of NAD(P)H in the cellular extracts (50 μl brought to 500 μl with TE buffer) was determined by fluorescence spectroscopy with a FluoroMax-3 (Horiba Jobin-Yvon) spectrofluorometer. Following excitation at 366 nm, emission spectra were recorded in the range 370 to 600 nm (maximum emission at 440 nm), and the amount of NAD(P)H (in μmol/liter) was determined on the basis of a titration curve obtained at known concentrations of commercial β-NADPH (Sigma) and normalized on the basis of the total protein content in the cellular extract.

RESULTS

Analysis of G6PDH in respiratory and fermentative mutants.

We reported the presence of an additional G6PDH activity band in cellular extracts from the K. lactis MW179-1D strain grown in ethanol (33). To further investigate this finding, we studied the G6PDH activity pattern in K. lactis respiratory mutants partially or totally impaired in ethanol utilization. Since these strains showed no or poor growth in minimal medium supplemented with ethanol, they were cultivated in ethanol-supplemented rich medium to study the effect of this carbon source on G6PDH. These mutants were deleted for KlNde1, KlNdi1, or KlCOX14, genes involved in the synthesis of components of the respiratory transport chain (Fig. 1). In particular, KlNde1 and KlNdi1 (35, 37, 38) are two transdehydrogenases located on the inner mitochondrial membrane, with the active site facing the outer membrane and the matrix, respectively. These two activities stem from single rotenone-insensitive proteins that in yeast substitute for the respiratory transport chain complex I of higher eukaryotes. Different from the corresponding enzymes of S. cerevisiae that use NADH as the substrate, KlNde1 but also KlNde2, a second transdehydrogenase similar to KlNde1, can reoxidize both NADH and NADPH (37, 38) (Fig. 1). The Klsdh1Δ and Klcox14Δ mutants unable to grow on respiratory carbon sources. Cell extracts were fractioned on polyacrylamide gels and stained for G6PDH activity. The values within the boxes in panels B and C indicate the amount (as a percentage) of each G6PDH band in each lane, as determined by densitometric analysis using the Molecular Imaging software (Kodak).

FIG 2 G6PDH activities separated on native gels for wild-type and mutant strains. (A) G6PDH from the MW179-1D strain (wt) and its isogenic respiratory mutants. (B) G6PDH from glycolytic and respiratory mutants in the CBS2359 context. (C) G6PDH from CBS2359 grown on increasing concentrations of glucose and from the MW179-1D strain transformed with the RAG1 multicopy plasmid. Extracts were prepared from late-exponential-phase cultures grown on YP medium containing glucose (D), ethanol (E), or glycerol (G) at 2% or at the concentration of glucose indicated in the figure. Glucose at 0.5% plus 2% ethanol (DE) was added to cultures of Klsdh1Δ and Klcox14Δ mutants unable to grow on respiratory carbon sources. Cell extracts were fractioned on polyacrylamide gels and stained for G6PDH activity. The values within the boxes in panels B and C indicate the amount (as a percentage) of each G6PDH band in each lane, as determined by densitometric analysis using the Molecular Imaging software (Kodak).

FIG 1 Glucose catabolic routes in wild-type (A) and adh null mutant (B) strains. The model depicts glycolysis, the PPP, mitochondrial respiratory transport chain, and major cytosolic NAD(P)red/NAD(P)H reoxidation activities. GPD1, NAD-dependent glycerol 3-P dehydrogenase; GUT2, FAD-dependent glycerol-3-P dehydrogenase; NDE1 and NDI1, external and internal inner mitochondrial membrane transdehydrogenases; PDC1, pyruvate decarboxylase; PG1, phosphoglucomutase; SDH, succinate dehydrogenase complex; Q, ubiquinone; bc1, complex III; cox, cytochrome oxidase. Pathways/activities involved in the accumulation/reoxidation of NAD(P)H are highlighted in boxes.
In the presence of ethanol, Klnde1Δ and Klslh1Δ showed two bands, as did the parental strain (Fig. 2A, lanes 4 and 8 versus lane 2), whereas Klnde1Δ and Klcox1Δ only showed the upper band of G6PDH activity (Fig. 2A, lanes 6 and 10). Since Klcox1Δ, differently from Kladh1Δ (32), is a respiratory-deficient mutant (11) and Klnde1Δ is devoid of the major NADH reoxidation activity (35), one can conclude that the expression of the second G6PDH band in the presence of ethanol requires respiration and efficient coenzyme reoxidation.

Extending such an analysis to other K. lactis reference strains, we obtained unexpected results. One of these strains, namely, CBS2359, in contrast with MW179-1D, showed both bands of activity in extracts from glucose cultures as well as from ethanol cultures (Fig. 2B, lanes 1 and 2), while showing only the upper band with glycerol (Fig. 2B, lane 3). To test the influence of fermentative and respiratory metabolism on the presence of the two activities, we analyzed three isogenic mutants impaired for those metabolisms. KlpdalΔ, one of these mutants, lacks the E1a subunit of the pyruvate dehydrogenase complex and, therefore, is unable to dissipate pyruvate through mitochondria (36, 42). This respiratory mutant showed a G6PDH activity pattern identical to that of the wild type (Fig. 2B, lanes 10 to 12), although both the wild type and KlpdalΔ mutant showed evidence of an elongated staining shadow below the faster-migrating G6PDH band (Fig. 2B, lanes 2, 10, and 11), suggesting an instability of this activity when present at higher levels. On the contrary, KlpglΔ and KlpdclΔ, two glycolytic mutants devoid of phosphoglucosomerase and the pyruvate decarboxylase activities, respectively (Fig. 1) (6, 13), when grown in the presence of glucose, expressed only the upper G6PDH band (Fig. 2B, lanes 4 and 7). Since these mutants showed the same pattern as MW179-1D (Fig. 2A, lane 1), we speculated that the slightly different kinetics of accumulation/oxidation of ethanol by CBS2359 and MW179-1D during fermentation (31, 35) (unpublished results) could be the basis for their different G6PDH patterns (Fig. 1A). To confirm this hypothesis, we analyzed the G6PDH pattern in CBS2359 cultures grown with increasing concentrations of glucose, i.e., progressively higher fermentative capabilities. Indeed, as shown in Fig. 2C, this strain had only one G6PDH band in samples from cultures containing glucose at concentrations below 0.6% (lanes 1 to 3). A faint, faster-migrating band of activity appeared with 0.6% glucose and increased in intensity when the glucose reached 4%, at which point the two bands were expressed at identical levels as determined by densitometric analysis (Fig. 2C, lanes 4 to 9). The link between accumulation/oxidation of ethanol during fermentation (Fig. 1A) and the presence of two G6PDH bands was also confirmed in the MW179-1D strain. In fact, the transformation of this strain with the plasmid containing the low-affinity glucose transporter RAG1 gene, which has been reported to increase glycolytic flux (22, 40), led to the appearance of the lower band of activity (Fig. 2C, lane 10). These results indicate that the presence of the faster-migrating band is related to the presence of ethanol, whether produced/oxidized during fermentation or added to the culture medium (Fig. 1A).

Analysis of G6PDH in adh mutants. Since alcohol dehydrogenases (ADH) are enzymes involved in the production and oxidation of ethanol (Fig. 1), we analyzed the G6PDH activity bands in extracts from cultures of adh mutants. In Fig. 3A is shown the G6PDH activity pattern from strain MW98-8C and its isogenic Kladh1Δ, Kladh2Δ, and Kladh4Δ mutants grown in glucose, ethanol, or glycerol medium. The parental strain has reduced fermentative capability (12) and, in line with previous findings, showed a single G6PDH band of activity in glucose (Fig. 3A, lane 1). The G6PDH patterns of the three adh mutants grown in ethanol or glycerol were identical to that of the parental strain (Fig. 3A, lanes 5 and 6, 8 and 9, and 11 and 12, versus lanes 2 and 3). Unexpectedly, all mutants displayed, though faint, a second band of activity also in glucose extracts (Fig. 3A, lanes 4, 7, and 10).

To test whether the number of ADH genes expressed in the cell influenced the presence of the two activity bands, we analyzed the G6PDH pattern in the adh° mutant. Indeed, as shown in Fig. 3B, the null strain, devoid of all ADH activities and unable to ferment (15), displayed the two bands of G6PDH (lane 1) as reported for the highly fermentative strain CBS2359. Moreover, the adh° mutant, unable to grow in minimal medium containing ethanol, still grew in rich ethanol medium (YPE); extracts prepared under this condition only showed the G6PDH upper band (Fig. 3B, lane 2). The important role played by ADH activities on the control of the G6PDH pattern was confirmed by the reintroduction of either KLAH1 or KLAH2 into the respective chromosomal locus of the adh° mutant (15), which allowed the reappearance of the lower G6PDH activity band with ethanol (Fig. 3B, lanes 5 and 8). However, the reintroduction of KLAH1 or KLAH2 did not lead to the disappearance of the faster-migrating G6PDH band in glucose-grown cells (Fig. 3B, lanes 4 and 7). On the contrary, the overexpression of KLAH1 and KLAH2 (data not shown) from a mul-
ticopy plasmid resulted in the presence of the G6PDH upper band alone in glucose or glycerol medium (Fig. 3B, lanes 10 and 12) and of two bands in ethanol (Fig. 3B, lane 11). These data confirmed that the intracellular amount of ADH activity is crucial for the appearance of one or both G6PDH bands when growth is carried out in glucose, while in ethanol even a small amount of ADH activity allows the expression of both bands (Fig. 3B, lanes 5, 8, and 11). Finally, the role of ADH in the control of G6PDH activity was confirmed by overexpressing in the CBS2359 strain other genes involved in the production and utilization of ethanol. These genes, KLAHL1, KLAHL3, KLAHL4, and KLAHL6, encode cytosolic KLAHL1 and KLAHL6 and mitochondrial KLAHL3 and KLAHL4 ADH and aldehyde dehydrogenase (ALD) activities, respectively (Fig. 1). As can be seen in Fig. 3C, the presence of both cytosolic activities altered the G6PDH glucose pattern of CBS2359, as the appearance of the lower band was inhibited (lanes 3 and 5). On the contrary, the mitochondrial KLAHL4 and KLAHL6 were unable to change the G6PDH pattern observed in the wild type (Fig. 3C, lanes 2 and 4 versus lane 1). Moreover, this role was limited to KLAHL1 and KLAHL6, in that the overexpression of other cytosolic or mitochondrial dehydrogenases, directly involved in the maintenance of the NAD(P)⁺/NAD(P)H redox balance, were unable to change the G6PDH expression pattern (Fig. 3C, lanes 6 to 10). These results indicate that the balance between cytosolic ADH and ALD activities and/or of the corresponding NAD⁺-NADH/NADP⁺-NADPH redox ratio control the appearance of the G6PDH faster-migrating band (Fig. 1).

NADP⁺ affects the G6PDH pattern in vitro. It has been reported that G6PDH activity is under strong feedback inhibition by NADPH (4), while NADP⁺ plays a major role in the tetramer-dimer interconversion of human G6PDH (41). To test in vitro the putative effect of NADP⁺(H) on G6PDH, we incubated the protein extracts, prepared from MW179-1D and CBS2359 glucose-grown cells, with increasing concentrations of NADP⁺(H). Cellular extracts of MW179-1D, expressing only the upper G6PDH band (Fig. 4A, lane 5), when incubated with NADP⁺ showed the appearance of the faster-migrating band at increasing concentrations of the cofactor (lanes 6 to 9). Moreover, when the same extract was assayed spectrophotometrically in the presence of 0.3 mM NADP⁺, in the absence of substrate, the appearance of a peak at 340 nm, indicative of NADPH formation, was observed (Fig. 4B, left panel). Since the incubation of NADP⁺ with the extract prepared from the MW179-1D/KlzwmΔ null strain gave no evidence for NADP⁺ reduction to NADPH (data not shown), we concluded that NADPH formation in the extract of MW179-1D was specifically due to G6PDH. Indeed, the absorbance at 340 nm was dependent on the NADP⁺ added to the cellular extract, with the initial velocities (v₀) following the Michaelis-Menten equation (Km = 0.029 ± 0.005 mM [mean ± standard deviation]) (Fig. 4B right panel). In contrast, incubation of the CBS2359 extracts with NADP⁺ showed no effect on the G6PDH activity pattern in either the native gel or absorption spectra (Fig. 4A, lanes 5 to 9, and C). The specific effect of NADP⁺ on the G6PDH activity pattern was further confirmed in both extracts from glycerol-grown CBS2359 cultures and from ethanol-grown MW179-1D cells, which expressed a single band of activity (Fig. 2B, lane 3) and two bands (Fig. 2A, lane 2), respectively. In fact, the incubation of glycerol-grown CBS2359 cell extract with NADP⁺ gave rise to the appearance of the faster-migrating G6PDH band in native gel, to the 340 nm peak of absorbance of NADPH, and to a variation of v₀ at increasing concentrations of NADP⁺ almost identical to those from the glucose-grown MW179-1D extract (data not shown). Conversely NADP⁺ was unable to modify the G6PDH activity pattern in the extract from ethanol-grown MW179-1D cells or the absorption spectra of the extract itself (data not shown). Notably, the effect of NADP⁺ was specific, since incubation of the extracts from either wild-type strain with NADPH showed no alteration of the G6PDH activity pattern (Fig. 4A, lanes 1 to 4). Therefore, the

FIG 4 (A) G6PDH activities separated on native gels following in vitro incubation with increasing concentration of NADPH and NADP⁺. The extracts (about 10 µg), prepared from MW179-1D and CBS2359 cultures grown on YPD, were incubated overnight on ice with the cofactors, fractioned on polyacrylamide gels, and stained for G6PDH. (B, left panel) Absorbance spectra from MW179-1D extract following the addition of 0.3 mM NADP⁺. (Right panel) Effect of concentration of NADP⁺ on the initial velocity of NADPH reduction. The solid line is the best fit, according to the Michaelis-Menten equation. (C) Absorbance spectra from CBS2359 extract following the addition of 0.3 mM NADP⁺. (D) An experiment as described for panel A but with extracts from an S. cerevisiae culture grown on YPD and human rhabdiosarcoma cell lines. The values in boxes beneath the gels indicate the percentage of each G6PDH band in each lane, as for Fig. 2.
appearance of the faster-migrating band in glucose-grown MW179-1D or glycerol-grown CBS2359 extracts could only be explained by the concomitant G6PDH-dependent reduction of NADP$^-$ to NADPH. It follows that the faster-migrating G6PDH band, evident in vivo in the glucose-grown CBS2359 extracts (Fig. 2C, lanes 4 to 9) or in ethanol-grown extracts from other strains, originates from the upper one following cytosolic accumulation of NADPH.

Because the amino acid sequences of G6PDH have been highly conserved during evolution (2,26), we generated, via the Swiss-Model server, two models of *K. lactis* G6PDH (data not shown), using as the templates the human enzyme (1,16, 28). Based on the homology model, the amino acid residues involved in tetramerization, in the binding of glucose 6-phosphate, coenzyme, and structural NADP$^-$ (Fig. 5), as well as in the dimerization (data not shown) appear fully conserved in nature and in space not only in *K. lactis* (Fig. 5) but also in *S. cerevisiae*. We therefore suggest that a common mechanism is involved in the control of the G6PDH isoforms in all eukaryotes. Thus, we also tested the effect of NADP$^-$ on *S. cerevisiae* and human G6PDH activities in vitro.

**FIG 5** Sequence alignment of G6PDH from *K. lactis* and *H. sapiens*, performed using Clustal X. The two proteins share 46% sequence identity. The fully conserved residues are shown by asterisks. The conservation of residues involved in the interaction with the coenzyme NADP$^-$ (inverted black triangle), the substrate glucose-6-phosphate (inverted white triangle), the structural NADP$^-$ (open circles), and in tetramerization (black circles) is shown. The structural model of *K. lactis* G6PDH was automatically built using as a template either one of the two crystal structures available for the human enzyme (PDB IDs 2BH9 or 1QKI). Both structures are from proteins missing the first 11 (1QKI) or the first 26 (2BH9) residues in the sequence.
under stress conditions to test whether the faster-migrating band available NADPH in the cell, the G6PDH patterns were analyzed. Thus, we assayed the NAD(P)H content in cell extracts from ethanol-grown cultures (two bands on the native gel) and from glycerol-grown cultures (a single G6PDH activity band on the native gel) in both the CBS2359 and MW179-1D strains. The amounts of the reduced cofactors in the extracts, determined for three independent samples under each growth condition, were performed by fluorescence measurements and were shown in both strains an NAD(P)H content 1.60 to 1.76 times higher in the ethanol-grown compared to glycerol-grown cell extracts.

**DISCUSSION**

G6PDH deficiency in humans is one of the most common enzypoathies; clinical symptoms associated with reduced activity are caused by the accumulation of toxic NADPH. In S. cerevisiae, inactivation of the G6PDH gene results in cell death. Therefore, the expression of this chimeric gene in the CBS2359 strain, harboring the Klzwf1Δ/GFP gene, could lead to the assembly of homo- and heterodimers or tetramers of the G6PDH and G6PDH/GFP activities. Indeed, cellular extracts from glycerol-grown cultures of CBS2359 harboring the Klzwf1Δ/GFP gene on a multicopy plasmid showed the presence of five different G6PDH bands of activity (Fig. 6, lanes 5 and 6) that, compared to the parental untransformed strain pattern (lane 8), were interpreted as homo- and heterotetramers of the Klzwf1Δ and Klzwf1Δ/GFP gene products (Fig. 6, lane 5, scheme). On the contrary, cellular extracts from ethanol cultures showed eight different G6PDH bands of activities in the transformed strain (Fig. 6, lanes 7, 10, and 11) and the expected two bands in the control strain (lane 9). The presence of three additional bands from ethanol-grown cells, compared to glycerol-grown cell extracts, could be assigned to the homodimers of G6PDH and G6PDH/GFP and to the heterodimer G6PDH-G6PDH/GFP (Fig. 5, lane 10, scheme). The amount of each G6PDH band, determined by densitometric analysis (Fig. 6, lanes 5 and 11), might reflect both the level of expression of the chimeric homo- and heterooligomeric activities.

The faster-migrating G6PDH band acts as a marker of cytosolic accumulation of NADPH. In S. cerevisiae, it has been reported that pgi1 mutants are unable to grow on glucose because diversion of the glycolytic flux toward the PPP leads to a toxic accumulation of NADPH that, by inhibiting G6PDH, may explain the observed cell growth arrest (7, 23). Moreover, in *Dicentrarchus labrax* liver, it has also been reported that accumulation of NADPH in the cytosol may lead to inhibition of G6PDH activity (4).

Since the main function of G6PDH is to guarantee readily available NADPH in the cell, the G6PDH patterns were analyzed under stress conditions to test whether the faster-migrating band of *K. lactis* could represent a sign of cytosolic accumulation of NADPH and/or of G6PDH inhibition. Therefore, CBS2359 and MW179-1D cultures were grown in the presence of hydrogen peroxide and acetaldehyde to induce oxidative stress that would require the NADPH produced in the PPP for the glutathione S-transferase (GSH)/glutaredoxin- and thioredoxin-dependent neutralization systems (9, 14, 24, 38). As shown in Fig. 7, the faster-migrating G6PDH activity band of CBS2359 decreased in intensity in the presence of H$_2$O$_2$ (lane 1) or in the presence of increasing concentrations of acetaldehyde (lanes 3 to 5), compared to the unstressed control cells (lane 2). On the contrary, the same compounds increased the amount of the single lower-migrating G6PDH band in strain MW179-1D (Fig. 7, lane 6 and 8 to 10). The latter finding suggests that G6PDH expression increases to better respond to the increased NADPH demands, compared to the unstressed control cells (Fig. 7, lane 7). Together, these results provide evidence that the appearance of the faster-migrating G6PDH is a direct consequence of NADPH accumulation in the cell, and it disappears when NADPH is rapidly consumed during oxidative stress.

In order to assess if there is a direct link between NAD(P)H accumulation and the presence of two activity bands of G6PDH, we assayed the NAD(P)H content in cell extracts from ethanol-grown cultures (two bands on the native gel) and from glycerol-grown cultures (a single G6PDH activity band on the native gel) in both the CBS2359 and MW179-1D strains. The amounts of the reduced cofactors in the extracts, determined for three independent samples under each growth condition, were performed by fluorescence measurements and were shown in both strains an NAD(P)H content 1.60 to 1.76 times higher in the ethanol-grown compared to glycerol-grown cell extracts.

**FIG 6** Chimeric G6PDH/GFP activities separated on native gels from the Klzwf1Δ and CBS2359. The strains were transformed with centromeric (for Klzwf1Δ) and multicopy (CBS2359) plasmids containing the indicated genes. Lanes 5 and 10 depict the assembly of oligomers; lanes 5 and 7, which are identical to lanes 6 and 10, respectively, contain a 2X amount of cell extract. Cultures were grown on YP containing glycerol (G) or ethanol (E). Staining for G6PDH and numberings to the right of lanes 5 and 11 were as described for Fig. 2.
hemolytic anemia, favism, and other pathologies caused by enhanced sensitivity of erythrocytes to oxidants (5). The native human G6PDH exists in a dimer/tetramer equilibrium, although the mechanism and/or physiological conditions that trigger this process are unknown. A better comprehension of these mechanisms will help to determine the regulatory means that control an activity essential for human life.

To test whether the two G6PDH activity-associated bands of K. lactis might correspond to the dimer and tetramer isoforms found with human G6PDH, we studied the metabolic conditions controlling their appearance. Since G6PDH in K. lactis is required for optimal growth on fermentative and respiratory carbon sources, with the exception of ethanol (33), the G6PDH pattern was analyzed in mutants altered for growth under both conditions.

Extensive analysis of these mutants showed that the ADH- and ALD-dependent cytosolic NAD(P)⁺/NAD(P)H redox ratio is the key factor affecting the appearance of the faster-migrating G6PDH band. In synthesis, the control of the G6PDH pattern is determined by the dynamic process involved in the production/oxidation of ethanol and acetaldehyde by ADH and ALD activities (Fig. 1A). The altered balance between these activities, as shown with the adh° mutant strain, leads to the presence of two G6PDH activity bands. This pattern can be explained by the activation of the KlAld6-dependent PDH bypass, which conveys the glucose flux toward NAD(P)H accumulation-dependent acetaldehyde and acetate production (42) (Fig. 1B).

Because cell growth is a dynamic process, the G6PDH pattern of the CBS2359 strain (Fig. 2C) has been used to explain the appearance of the faster-migrating activity band. In respiratory growth cultures (D, 0.1 to 0.4%), in which ethanol was not produced (31), we observed the presence of a single G6PDH band (Fig. 2C, lanes 1 to 3). Under these conditions, the glycolytic and the PPP accumulated NADH and NADPH in the cytosol. The reduced cofactors are then transferred to the respiratory chain, by the ethanol/acetaldehyde and glyceraldehyde-3-phosphate shuttles (27, 33, 34) and the transdehydrogenase KlNde1 (35, 37), where they are reoxidized (Fig. 1). In cultures containing glucose at >0.4%, the progressive accumulation of ethanol, which is linked to the reduced oxygen availability, leads to a concomitant increase of the faster-migrating G6PDH band (Fig. 2C, lanes 4 to 9). In fact, in Crabtree-negative yeast, like K. lactis (10), fermentation can only occur under oxygen limitation (17), while the produced ethanol could be oxidized contemporarily to glucose, leading to the accumulation of both reduced cofactors (Fig. 1A). Since only the mitochondrial transdehydrogenase KlNde1 can directly oxidize NADPH and NADH (37), these cofactors compete for KlNde1 for their reoxidation. Overkamp et al. (27) reported that isolated mitochondria from CBS2359 showed NADH-dependent oxygen consumption rates that were twice that of NADPH. Therefore, KlNde1 having a higher affinity for the former, accepts preferentially NADH as the substrate, leading to the accumulation of NADPH in the cytoplasm. All our data indicate that the accumulation of NAD(P)H during fermentation/growth on ethanol is tightly bound to the appearance of the faster-migrating band, which has an inhibitory effect on the wild-type biomass yield, as suggested by the better growth of the Klzwf1Δ mutant on ethanol medium, because the absence of KlzWF1 leads to a reduced accumulation of NADPH (33). On the contrary, higher intracellular NADPH demand, as occurs under stress conditions, led to the specific disappearance of the faster-migrating G6PDH band (Fig. 7), confirming its role as an inhibitory component of G6PDH to avoid toxic accumulation of NADPH (4).

In fact, for S. cerevisiae, in which NADPH accumulation is toxic/inhibitory for cell growth, as reported for the pgi1 mutant (7), cells are unable to grow on glucose. Since Nde1 and Nde2 can only accept NADH as the substrate (23), we observed a single G6PDH band of activity under all growth conditions (33). It follows that the NADPH-dependent regulation of G6PDH observed in K. lactis is not operative under physiological conditions in S. cerevisiae, but as shown in vitro assays (Fig. 4D), the evolutionarily conserved amino acid structure of this enzyme (Fig. 5) suggests a common regulatory mechanism in all eukaryotes.

Crystal structure and biochemical analyses of human G6PDH have shown that each subunit of this enzyme binds, well separated from the catalytic coenzyme binding site, a structural molecule of NADP⁺, between the dimers (3, 19, 39). We propose that the replacement of NADP⁺ with NADPH causes a conformational change and/or a variation in the net charge/oligomeric state of K. lactis G6PDH that leads to the appearance of a new G6PDH band with faster migrating properties. Eight different G6PDH multimers have been observed in K. lactis extracts from ethanol-grown cultures with the contemporary expression of KLZWF1 and KLZWF1GFP gene products, indicating that upon binding/loss of NADPH from the structural site, a conformational change and partial interconversion of the tetramer into dimers occur (Fig. 6).

Indeed, it has been reported that G6PDH, carefully stripped of its structural NADP⁺, is both active and dimeric (39), as we also showed for native G6PDH from human cell lines (Fig. 4D, lanes 9 to 11). These results, reported for purified human enzyme, fit very well with the presence of the two isoforms in humans after incubation with NADP⁺ (Fig. 4D, lanes 15 to 18) and also in K. lactis, in which the faster-migrating band/dimer rarely exceeds 50% of the total amount of G6PDH. Its abundance in the cell is probably a niche-specific genetic trait of K. lactis that is influenced by the NAD⁺(H)/NADP⁺(H) dynamic balance as determined by the levels of respiration/fermentation, the requirement for NADPH-dependent detoxifying activities, and the differential affinities of KlNde1 for NADH and NADPH. Because these cofactors are necessary for energy metabolism (NADH) and the anabolism/stress response (NADPH), we can speculate that G6PDH inhibition by NADPH in excess helps to distribute the glucose flux between glycolysis and PPP according to the cell’s needs. The involvement of the NAD⁺(H)/NADP⁺(H) redox ratio also in the regulation of the S. cerevisiae GAL induction system has been proposed by R. Kumar et al. (21). In fact, a similarity exists between the regulation of two G6PDH isoforms and the Gal4-Gal80 complex, since both seem to require NADP⁺ molecules to trigger their circuits. In the present work, we also found that analysis of human G6PDH by native gel electrophoresis can be exploited to determine the redox state in tissues and cell lines under physiological and pathological conditions.

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