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 NAD$^+$ (H)/NADP$^+$ (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 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.

**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 KIZWF1GFP plasmids.** The entire KIZWF1 gene, excised from pTZ19/KIZWF1 (33) as a HindIII/Xbal fragment, was cloned into the multicopy pKL plasmid to harbor pKL-KIZWF1. pKL is a Geneticin resistance pKD1-derived stable multicopy vector (31). This plasmid was also used for cloning and over-expression of genes amplified by PCR from the *K. lactis* genome. The primers used for the amplification of KIALD4, KIALD6, and KIGPD1 and for the construction of the chimeric KIZWF1GFP genes are reported in Table 1, while those for KIGUT2, KINDE1, and KINDII have been reported elsewhere (34, 35). KIZWF1GFP was constructed by amplifying the 5’ portion (980 bp of the promoter plus the entire KIZWF1 open reading frame [ORF] without the stop codon) and the 3’ portion (stop codon and
Table 1: Yeast strains and DNA primers used in this study

| Strain or target genes and primers | Genotype or sequence | Reference or source |
|-----------------------------------|----------------------|---------------------|
| **K. lactis strains**             |                      |                     |
| CBS2359<sup>a</sup>              | MATa                 | CBS collection      |
| GG1993                            | CBS2359 Klpcda::Tn5BLE |                     |
| GG1996                            | CBS2359 Klpg1::loxP (rag2Δ) |                     |
| CBS2359/152F<sup>a</sup>         | MATa metA1 ura3      | 29                  |
| CBS2359/152F/2                    | CBS2359 Klpcd1::URA3 metA1 ura3 | 29                  |
| MW98-8C                           | Matα lysA1 argA1 ura3 rag1 rag2 adh3 | 12                  |
| CF1                               | MW98-8C Kladh1::URA3  | 25                  |
| CF2                               | MW98-8C Kladh2::URA3  | 25                  |
| MW179-1D                          | Matα metA1 ade-T600 leu2 trpA1 ura3 lac4 | 11                  |
| MW179-1D/Kcox14Δ                  | MW179-1D Kcox4::KanMX4 | 11                  |
| MW179-1D/Kldel1Δ                  | MW179-1D Klde1::KanMX4 | 35                  |
| MW179-1D/Kldid1Δ                  | MW179-1D Klid1::KanMX4 | 35                  |
| MW179-1D/Kldsh1Δ                  | MW179-1D Klsh1::KanMX4 | 32                  |
| MW179-1D/Klszf1Δ                  | MW179-1D Klzf1::KanMX4 | 33                  |
| MS7-62<sup>a</sup>                | Matα lysA1 argA1 Kladh1Δ Kladh2Δ Kladh3Δ Kladh4Δ | 30                  |
| MS7-62/KlADH1                     | Matα lysA1 argA1 Kladh2Δ Kladh3Δ Kladh4Δ | 15                  |
| MS7-62/KlADH2                     | Matα lysA1 argA1 Kladh1Δ Kladh2Δ Kladh3Δ Kladh4Δ | 15                  |
| BY4741                            | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf collection |

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

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

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 EcoRV, a unique site located before the stop codon, and ligated with the EcoCR 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) (N0505 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% CO2, 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 onto 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 of TE 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 a ultrafiltration devices with a cutoff 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 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, KNDe1 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).
In the presence of ethanol, Kl\textit{ndi1}\Delta and Kl\textit{sdh1}\Delta showed two bands, as did the parental strain (Fig. 2A, lanes 4 and 8 versus lane 2), whereas Kl\textit{ndi1}\Delta and Kl\textit{cox1}\Delta only showed the upper band of G6PDH activity (Fig. 2A, lanes 6 and 10). Since Kl\textit{cox1}\Delta, differently from Kl\textit{adh1}\Delta (32), is a respiratory-deficient mutant (11) and Kl\textit{ndi1}\Delta 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\textit{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. Kl\textit{pld1}\Delta, one of these mutants, lacks the E1\alpha 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 Kl\textit{pld1}\Delta 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, Kl\textit{pgi1}\Delta and Kl\textit{pdc1}\Delta, two glycolytic mutants devoid of phosphoglucoisomerase 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, 33) (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 \textit{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 \textit{adh} mutants. In Fig. 3A is shown the G6PDH activity pattern from strain MW98-8C and its isogenic Kl\textit{adh1}\Delta, Kl\textit{adh2}\Delta, and Kl\textit{adh4}\Delta 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 \textit{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 \textit{ADH} genes expressed in the cell influenced the presence of the two activity bands, we analyzed the G6PDH pattern in the \textit{adh}° mutant. Indeed, as shown in Fig. 3B, the null strain, devoid of all \textit{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 \textit{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 \textit{ADH} activities on the control of the G6PDH pattern was confirmed by the reintroduction of either \textit{KIAH1} or \textit{KIAH2} into the respective chromosomal locus of the \textit{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 \textit{KIAH1} or \textit{KIAH2} 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 \textit{KIAH1} and \textit{KIAH2} (data not shown) from a multicyclic plasmid containing the genes indicated in each lane. Cell growth, cell extracts, gel conditions, and staining for G6PDH, as well as the values in the boxes, are as those described for Fig. 2.
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, KlADH1, KlADH3, KlALD4, and KlALD6, encode cytosolic (KlAdh1 and KlAdh6) and mitochondrial (KlAdh3 and KlALD4) 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 KlALD4 and KlAdh3 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 KlAdh1 and KlALD6, 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/NAD⁺⁺-NADPH redox ratio control the appearance of the G6PDH faster-migrating band (Fig. 1).

NAD⁺⁺ affects the G6PDH pattern in vitro. It has been reported that G6PDH activity is under strong feedback inhibition by NADPH (4), while NAD⁺⁺ 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/Klzfw1Δ 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 (vₒ) following the Michaelis-Menten equation (Kᵥ = 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 vₒ 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...
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 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 NADPH/H (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 NADPH on S. cerevisiae and human G6PDH activities in vitro. Cellular extracts prepared from S. cerevisiae and from human rhabdomyosarcoma cells (a cancer made up of cells that normally develop into skeletal muscles) expressed a single band of G6PDH (Fig. 4D, lanes 1 and lanes 9 to 11, respectively). However, when incubated with increasing concentrations of NADPH, these extracts showed the presence of two bands (Fig. 4D, lanes 5 to 8 and 14 to 18) that were clearly visible in both extracts, starting from 6 mM NADPH (Fig. 4D, lanes 5 and 15). This concentration of the cofactor was similar to that responsible for the appearance of the faster-migrating G6PDH band in K. lactis (Fig. 4A, lanes 6 and 7).

Interestingly, the addition of NADPH to human extracts produced a stabilizing effect on the two G6PDH activities compared to the extract incubated without cofactor or with just small amounts of cofactor (Fig. 4C, lanes 13 to 18 versus lane 12). Moreover, the single band present in human extract (Fig. 4D, lanes 9 to 12) seemed to correspond to the faster-migrating band when compared to K. lactis and S. cerevisiae G6PDH bands. K. lactis G6PDH bands correspond to the tetramer-dimer isoforms of human activity.

The role played by NADPH in the appearance of the second G6PDH band in yeasts and humans and the high conservation in amino acid residues responsible for G6PDH activity and structural integrity (2, 26) suggested that the two native bands detected in K. lactis corresponded to the dimer and tetramer G6PDH forms observed in the human enzyme. Dur-

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 NADPH (inverted black triangle), the substrate glucose-6-phosphate (inverted white triangle), the structural NADPH (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.
ing expression of the chimeric Klzwf1Δ gene in the Klzwf1Δ mutant, we noticed not only that the chimeric G6PDH/GFP activities restored the growth defects of the mutant (data not shown), but also that cellular extracts prepared from these transformed cultures showed the presence of G6PDH activity bands with reduced migrating properties, compared to the pattern of the null strain transformed with the chimeric Klzwf1 gene (Fig. 6, lanes 1 to 4). Therefore, the expression of this chimeric gene in the CBS2359 strain, harboring Klzwf1 on its chromosomal locus, 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 KlzWF1/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 KlzWF1 and KlzWF1/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 chromosomal and plasmidic gene but also the differential stability of the wild type compared to the chimeric homo- and hetero-oligomeric 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 H2O2 (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, lanes 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 cellular 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 showed 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 enzymopathies; clinical symptoms associated with reduced activity are...
hemolytic anemia, favism, and other pathologies caused by enhanced sensitivity of erythrocytes to oxidants (5). The native human 6PGDH 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 6PGDH activity-associated bands of *K. lactis* might correspond to the dimer and tetramer isoforms found with human 6PGDH, we studied the metabolic conditions controlling their appearance. Since 6PGDH in *K. lactis* is required for optimal growth on fermentative and respiratory carbon sources, with the exception of ethanol (33), the 6PGDH 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 6PGDH band. In synthesis, the control of the 6PGDH 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 *adl*⁺ mutant strain, leads to the presence of two 6PGDH activity bands. This pattern can be explained by the activation of the KIAld6-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 6PGDH 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 6PGDH 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 transhydrogenase 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 band/dimer rarely exceeds 50% of the total amount of 6PGDH. Its abundance in the cell is probably a niche-specific genetic trait of *K. lactis* that leads to the appearance of a new 6PGDH band with faster migrating properties. Eight different 6PGDH multimers have been observed in *K. lactis* extracts from ethanol-grown cultures with the contemporary expression of KIZWF1 and KIZWF1GFP 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 6PGDH, carefully stripped of its structural NADP⁺, is both active and dimeric (39), as we also showed for native 6PGDH 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 6PGDH. 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 6PGDH 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 6PGDH 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 6PGDH by native gel electrophoresis can be exploited to determine the redox state in tissues and cell lines under physiological and pathological conditions.

**ACKNOWLEDGMENTS**

This work was funded by PRIN 2009 and by “Ateneo 2010,” Sapienza University of Rome.

We thank Sirio D’Amici for technical help.
REFERENCES

1. Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The Swiss-Model workspace: a web-based environment for protein structure homology modeling. Bioinformatics 22:195–201.

2. Au SW, et al. 1999. Solution of the structure of tetrmeric human glucose 6-phosphate dehydrogenase by molecular replacement. Acta Crystallogr. D Biol. Crystallogr. 55:826–834.

3. Au SW, Gower S, Lam VM, Adams MJ. 2000. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP+ molecule and provides insights into enzyme deficiency. Structure 8:293–303.

4. Bautista JM, Garrido-Perttierra A, Soler G. 1988. Glucose-6-phosphate dehydrogenase from Dictyotenchus labrux liver: kinetic mechanism and kinetics of NADPH inhibition. Biochim. Biophys. Acta 967:354–363.

5. Beutler E. 1994. G6PD deficiency. Blood 84:3613–3636.

6. Bianchi MM, Tizzani L, Destrueille M, Frontali L, Wesolowski-Louvel M. 1996. The petite-negative yeast Kluyveromyces lactis has a single gene expressing pyruvate decarboxylase activity. Mol. Microbiol. 19:27–36.

7. Boles E, Lehnter W, Zimmermann FK. 1993. The role of the NADP-dependent glutamate dehydrogenase in restoring growth on glucose of a Saccharomyces cerevisiae phosphoglucone isomerase mutant. Eur. J. Biochem. 217:469–477.

8. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. Anal. Biochem. 72:248–254.

9. Carmel-Harel O, Storz G. 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and Saccharomyces cerevisiae responses to oxidative stress. Annu. Rev. Microbiol. 54:439–461.

10. De Deken RH. 1966. The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol. 44:149–156.

11. Fiori A, Saliola M, Goffrini P, Falcone C. 2000. Isolation and molecular characterization of KICOX14, a gene of Kluyveromyces lactis encoding a protein necessary for the assembly of the cytochrome oxidase complex. Yeast 16:307–314.

12. Goffrini P, Algeri AA, Donnini C, Wesolowski-Louvel M, Ferrero I. 1989. RAG1 and RAG2: nuclear genes involved in the dependence/independence on mitochondrial respiratory function for growth on sugar. Yeast 5:99–105.

13. Goffrini P, Wesolowski-Louvel M, Ferrero I. 1991. A phosphoglucone isomerase gene is involved in the Rag phenotype of the yeast Kluyveromyces lactis. Mol. Gen. Genet. 228:401–409.

14. Iwasa S, Maeda K, Miki T, Inoue Y, Kimura A. 2000. Human mutations in glucose-6-phosphate dehydrogenase reflect evolutionary history. FASEB J. 14: 485–494.

15. Izawa S, Maeda K, Miki T, Inoue Y, Kimura A. 2000. Human mutations in glucose-6-phosphate dehydrogenase reflect evolutionary history. FASEB J. 14: 485–494.

16. Kletzien RF, Harris PKW, Foellmi LA. 1993. Human mutations in glucose-6-phosphate dehydrogenase reflect evolutionary history. FASEB J. 14: 485–494.

17. Kiefer F, Arnold K, Künzli M, Bordoli L, Schwede T. 2009. The Swiss-Model repository and associated resources. Nucleic Acids Res. 37:387–392.

18. Kiers J, et al. 1998. Regulation of alcoholic fermentation in batch and chemostat cultures of Kluyveromyces lactis CBS2359. Yeast 14:459–469.

19. Kletzien RF, Harris PKW, Foellmi LA. 1994. Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J. 8:174–181.

20. Kotaka M, et al. 2005. Structural studies of glucose-6-phosphate and NADP+ binding to human glucose-6-phosphate dehydrogenase. Acta Crystallogr. D 61:495–504.

21. Kumar PR, Yu Y, Sternglaz R, Johnston SA, Joshua-Tor L. 2008. NADP regulates the yeast GAL induction system. Science 319:1090–1092.

22. Lemaire M, Wesolowski-Louvel M. 2004. Enolase and glycolytic flux play a role in the regulation of the glucose permease gene RAG1 of Kluyveromyces lactis. Genetics 168:723–731.

23. Lutik MA, et al. 1998. The Saccharomyces cerevisiae NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J. Biol. Chem. 273:24529–24534.

24. Matsufuji Y, et al. 2008. Acatelaldehyde tolerance in Saccharomyces cerevisiae involves the pentose phosphate pathway and oleic acid biosynthesis. Yeast 25:825–833.

25. Mazzoni C, Saliola M, Falcone C. 1992. Ethanol-induced and glucose-insensitive alcohol dehydrogenase in the yeast Kluyveromyces lactis. Mol. Microbiol. 6:2279–2286.

26. Notaro R, Afolayan A, Luzzatto L. 2000. Human mutations in glucose-6-phosphate dehydrogenase reflect evolutionary history. FASEB J. 14: 485–494.

27. Overkamp KM, Bakker BM, Steensma HY, van Dijken JP, Pronk JT. 2002. Two mechanisms for oxidation of cytosolic NADPH by Kluyveromyces lactis mitochondria. Yeast 19:813–824.

28. Peitsch MC. 1995. Protein modeling by E-mail. Biotechnology 13:658–660.

29. Salani F, Bianchi M. 2006. Production of glucoamylase in pyruvate decarboxylase deletion mutants of the yeast Kluyveromyces lactis. Appl. Microbiol. Biotechnol. 69:564–572.

30. Saliola M, Bellardi S, Marta I, Falcone C. 1994. Glucose metabolism and ethanol production in adh multiple and null mutants of Kluyveromyces lactis. Yeast 10:1133–1140.

31. Saliola M, Falcone C. 1995. Two mitochondrial alcohol dehydrogenase activities of Kluyveromyces lactis are differentially expressed during respiration and fermentation. Mol. Gen. Genet. 249:665–672.

32. Saliola M, Bartoccioli PC, De Maria I, Lodi T, Falcone C. 2004. The deletion of the succinate dehydrogenase gene KISDH1, in Kluyveromyces lactis, does not lead to respiratory deficiency. Eukaryot. Cell 3:589–597.

33. Saliola M, et al. 2007. Deletion of the glucose-6-phosphate dehydrogenase gene KIZWP1 affects both fermentative and respiratory metabolism in Kluyveromyces lactis. Eukaryot. Cell 6:19–27.

34. Saliola M, Sponzillo ML, D’Amici S, Lodi T, Falcone C. 2008. Characterization of KIGUT2, a gene of the glycerol-3-phosphate shuttle, in Kluyveromyces lactis. FEMS Yeast Res. 8:697–705.

35. Saliola M, et al. 2010. The transdehydrogenase genes KINDE1 and KINDE1 regulate the expression of KIGUT2 in the yeast Kluyveromyces lactis. FEMS Yeast Res. 10:518–526.

36. Steensma HY, Ter Linde JJM. 2001. Plasmids with the Cre recombinase and the dominant nat marker, suitable for use in prototrophic strains of Saccharomyces cerevisiae and Kluyveromyces lactis. Yeast 18:469–472.

37. Tarro N, Diaz Prado S, Cerdan ME, Gonzalez Siso MI. 2005. The nuclear genes encoding the internal (KINDEI) and external (KINDEII) alternative NAD(P)H:ubiquinone oxidoreductases of mitochondria from Kluyveromyces lactis. Biochim. Biophys. Acta 1707:199–210.

38. Tarro N, Becerra M, Cerdan ME, Gonzalez Siso MI. 2006. Reoxidation of cytosolic NADPH in Kluyveromyces lactis. FEMS Yeast Res. 6:371–380.

39. Wang X-T, Chan TF, Lam VMS, Engel PC. 2008. What is the role of the second structural NADP+–binding site in human glucose 6-phosphate dehydrogenase? Protein Sci. 17:1403–1411.

40. Wesolowski-Louvel M, Goffrini P, Ferrero Fukuhara H. 1992. Glucose transport in the yeast Kluyveromyces lactis. I. Properties of an inducible low-affinity glucose transporter. Mol. Gen. Genet. 233:89–96.

41. Wrigley NG, Heath JV, Bonsignore A, De Flora A. 1972. Human erythrocyte glucose 6-phosphate dehydrogenase: electron microscope studies on structure and interconversion of tetramers, dimers and monomers. J. Mol. Biol. 68:483–499.

42. Zeeman AM, et al. 1998. Inactivation of the Kluyveromyces lactis KIPDA1 gene leads to loss of pyruvate dehydrogenase activity, impairs growth on glucose and triggers aerobic alcoholic fermentation. Microbiology 144: 3437–3446.