The Deletion of the Succinate Dehydrogenase Gene \textit{KlSDH1} in \textit{Kluyveromyces lactis} Does Not Lead to Respiratory Deficiency

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We have isolated a \textit{Kluyveromyces lactis} mutant unable to grow on all respiratory carbon sources with the exception of lactate. Functional complementation of this mutant led to the isolation of \textit{KlSDH1}, the gene encoding the flavoprotein subunit of the succinate dehydrogenase (SDH) complex, which is essential for the aerobic utilization of carbon sources. Despite the high sequence conservation of the \textit{SDH} genes in \textit{Saccharomyces cerevisiae} and \textit{K. lactis}, they do not have the same relevance in the metabolism of the two yeasts. In fact, unlike \textit{SDH1}, \textit{KlSDH1} was highly expressed under both fermentative and nonfermentative conditions. In addition to this, but in contrast with \textit{S. cerevisiae}, \textit{K. lactis} strains lacking \textit{KlSDH1} were still able to grow in the presence of lactate. In these mutants, oxygen consumption was one-eighth that of the wild type in the presence of lactate and was normal with glucose and ethanol, indicating that the respiratory chain was fully functional.

Northern analysis suggested that alternative pathway(s), which involves pyruvate dehydrogenase and the glyoxylate cycle, could overcome the absence of SDH and allow (i) lactate utilization and (ii) the accumulation of succinate instead of ethanol during growth on glucose.

Succinate dehydrogenase (SDH) is a component of complex II of the respiratory chain that catalyses the oxidation of succinate in the Krebs cycle and feeds electrons to the ubiquinone pool. The complex, which is highly conserved through evolution, is located in the inner mitochondrial membrane and consists of two catalytic and two structural subunits, all encoded by nuclear genes (38). In \textit{Saccharomyces cerevisiae}, the four genes (\textit{SDH1} to \textit{SDH4}) coding for SDH have been isolated and characterized (26, 27, 45, 47). The flavoprotein subunit (11, 42) responsible for the oxidation of succinate to fumarate is encoded by two paralogous genes, \textit{SDH1} and \textit{SDH1b}, although only \textit{SDH1} is necessary for growth on respiratory carbon sources (11). \textit{SDH2} codes for the iron-protein subunit (31) that contains three different iron-sulfur centers (22) and, together with the protein Sdh1p, constitutes the catalytic core of the SDH complex, which conveys electrons from the covalently attached flavin adenine dinucleotide (FAD) of Sdh1p first to the iron-sulfur centers and then to ubiquinone. \textit{SDH3} and \textit{SDH4} code for two small hydrophobic peptides, which anchor the complex to the inner mitochondrial membrane (10, 15). In humans, the mutations in the \textit{SDH} genes have been associated to several mitochondrial-related pathologies suggesting, beside the enzymatic activity of the complex in the Krebs cycle, its involvement in superoxide handling (39, 43).

In \textit{S. cerevisiae}, the expression of the \textit{SDH} genes is repressed by glucose and derepressed on respiratory carbon sources (31, 45), and the loss of SDH functions results in the inability of cells to grow on any respiratory carbon sources (12, 47).

In this paper we report the isolation of the \textit{KlSDH1} gene (EMBL accession number AJ555233) encoding the \textit{Kluyveromyces lactis} flavoprotein subunit of the SDH complex. We show that, despite the general sequence conservation between \textit{K. lactis} and \textit{S. cerevisiae} genes, their regulation appears to be different, probably reflecting the predominant respiratory and fermentative nature, respectively, of these species (18, 51). The genes are expressed on both fermentable and nonfermentable carbon sources, and their deletion does not lead to a loss of the respiratory function.

MATERIALS AND METHODS

Strains, media and culture conditions. The strains used in this work are reported in Table 1. Yeast cultures were grown overnight under aerated conditions on an orbital shaker at 28°C inYP medium (1% Difco yeast extract, 2% Difco Bacto-peptone) or in minimal medium (0.7 g of Difco yeast nitrogen base per liter), supplemented with different carbon sources at the concentrations specified in the text. Solid media were supplemented with 2% Bacto agar (Difco). Curve growth was performed by inoculating about 10⁶ cells per ml of culture medium, and at time intervals aliquots of the cultures were taken, suitably diluted, and counted in a Thoma chamber to determine cell concentration (cells/milliliter). Minimal media were supplemented with the required auxotrophies at a final concentration of 10 μg/ml.

\textit{Escherichia coli} strain DH5α was used for the propagation of plasmid DNA. Plasmid-carrying bacteria were grown at 37°C on LB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl) supplemented with 100 μg of ampicillin per ml.

Ethanol, glucose, and succinate concentrations in culture supernatants in culture supernatants were measured by HPLC. 

\textit{KlSDH1} disrupting cassette. The plasmid p3AS, containing the complementing fragment (about 4.6 kbp, plus 0.2 kbp at the SphI site) in the Kep6 multicopy vector (5), was used for the construction of the disrupting cassette (see Fig. 3 for transformants). About 80% of the \textit{KlSDH1} open reading frame (XbaI-BglII fragment of 1.5 kbp) was replaced with the genes \textit{kanMX4} (49) and \textit{URA3} of \textit{S. cerevisiae}. The wild-type \textit{MW179} 1D strain was transformed with the linearized cassette to uracil prototroph and resistance to geneticin (G418). Positive clones were replica plated on minimal media containing glyceral to identify those
impaired in growth. These clones were further analyzed by Southern blotting to verify the correct integration of the cassette into the K. lactis locus.

**SDH assay on electrophoresis gels.** Cell extracts for the SDH staining assay were prepared in the following way. Cultures were grown to the early stationary phase in 20 ml of YP or 100 ml of minimal medium containing 2% glucose. Cells were collected, washed with 0.6 M sorbitol, and resuspended in 300 μl of TE-sorb (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.6 M sorbitol), and cold glass beads were added at about two-thirds the final solution volume (diameter, 0.5 mm; B. Braun Melsungen AG). Cell extracts were prepared by vortexing samples in micro tubes for 3 to 4 min in a refrigerator. Cell debris was pelleted for 5 min at 4,000 rpm in a bench top centrifuge (Sigma 1-26) and discarded. Supernatants were collected and centrifuged for another 30 min at 15,000 to 20,000 rpm. The cytoplasmic supernatant was kept and used as a control for an SDH native staining assay, and the mitochondrial pellet was washed once with 0.5 μl of TE-Sorb and centrifuged for another 30 min at 15,000 to 20,000 rpm. The supernatant was discarded, and the final pellet was resuspended in 15 μl of TE-triton (10 mM TRIS-HCl [pH 7.5], 1 mM EDTA, 0.2% Triton X-100) plus 15 μl of 4× loading buffer (0.1 M Tris-HCl [pH 6.8], 50% glycerol, 0.02 M β-mercaptoethanol, 0.008% bromphenol blue) and dispensed mechanically by repeated pipetting. A total of 25 μl of the mixture was loaded on 5% polyacrylamide native gel (Amresco) prepared as previously described (33, 34, 44). Supernatant (25 μl) was used as a cytoplasmic SDH staining control. The gel was run in a refrigerator in a mini-apparatus (Hoefer Scientific Instruments), at 20 mA constant current until the dye reached the bottom of the gel (about 1 h). The SDH staining mixture for one gel (5 ml) contains the following: 15 μl of phenazine methosulfate (catalogue no. P-9625; Sigma), equivalent to a concentration of 40 mg/ml in distilled water; 30 μl of nitro blue tetrazolium (product no. 590 SALIOLA ET AL. EUKARYOT. CELL

| Strain          | Genotype                  | Reference or source |
|-----------------|---------------------------|---------------------|
| K. lactis strains |                           |                     |
| MS14-1A         | MATa, ade, tripl, ura3, sdh | This work           |
| MW278-20C/1     | MATa, ade2, leu2, ura3, lac4-8 | 17                  |
| MW179-1D        | MATa, metA1, ade2, tripl1, ura3, leu2, lac4-8 | 17                  |
| MW179-1D/Klsdh1Δ | MW179-1D klsdh1::kanMX4URA3 | This work           |
| CBS2359/152     | MATa, metA1               | 50                  |
| GGI933          | MATa, Klpld1::Tn5BLE, ura3-49 | 54                  |
| S. cerevisiae strains |                       |                     |
| BY4741          | MATa, his3Δ1, leu2α20, met15Δ0, ura3Δ0 | Eurosarf           |
| BY4741sdh1Δ     | BY4741 sdh1::kanMX4       | Eurosarf            |
| BY4741sdh1Δ     | BY4741 sdh1b::kanMX4      | Eurosarf            |

* Isogenic derivative of CBS2359

**RESULTS**

**Isolation of the KISDH1.** During a screening of K. lactis mutants affected in the respiratory metabolism, we isolated MS14-1A, a mutant that failed to grow on minimal medium containing respiratory carbon sources such as glycerol (Fig. 1), acetate, ethanol, pyruvate and succinate (data not shown), but it was still able to grow in the same medium containing lactate (see Fig. 6). We performed the genetic analysis of this strain by crossing it with the wild-type strain CBS2359/152. The presence of a single mutation in the MS14-1A strain was demonstrated by the meiotic 2+2 segregation of the growth phenotype on minimal medium containing glycerol in a functional complementation experiment. MS14-1A was transformed with a genomic library in the Kep6 multicopy vector (5). Out of

![Fig. 1. Growth was compared between strains MW179-1D (1), MS14-1A (2), and MS14-1A (3) complemented with the multicopy plasmid containing the KISDH1 gene. The plates are minimal media containing glycerol or glucose as carbon sources.](image-url)
14,000 Ura\(^+\) transformants analyzed, two showed a growth rate comparable to that of the wild-type reference strains (Fig. 1). Restriction analysis of the plasmids recovered from the two clones showed identical DNA inserts of 6.1 kbp. By deletion, we reduced the complementing region to a fragment of about 4.6 kbp. Sequence analysis showed the presence in this fragment of an open reading frame of 651 codons located between the SspI sites (see Fig. 3). A deduced amino acid sequence comparison revealed a significant degree of identity with the genes coding for the flavoprotein subunit of the SDH complex from various organisms (7, 11, 24, 42, 52). As shown in Fig. 2, a multiple protein alignment showed high sequence conservation along the entire protein between the Klsdh1p and other Sdh1 proteins. The \textit{K. lactis} protein was 52\% identical to that of \textit{E. coli}, 62\% identical to the \textit{Homo sapiens} protein, and 84 and 79\% identical to the \textit{S. cerevisiae} Sdh1 and Sdh1b proteins, respectively. There was a higher degree of identity if we exclude the first 56 amino acids, which represent the mitochondrial targeting sequence that is absent in the \textit{E. coli} protein (11, 40). We therefore called the \textit{K. lactis} gene \textit{KlSDH1}.

**Construction of the \textit{KlSDH1} null mutant.** In \textit{S. cerevisiae}, strains lacking \textit{SDH1} are unable to grow on nonfermentable carbon sources while \textit{SDH1b} is slightly expressed and dispensable (13, 47). To study the effect of the deletion of \textit{KlSDH1} in \textit{K. lactis} cells, we constructed a \textit{Klsdh1} disrupted mutant. Southern analysis (Fig. 3C) confirmed the correct integration of the cassette at the \textit{KlSDH1} locus. In fact, the wild-type 4.4-kbp fragment (lane 1) was replaced, as expected, by a 5.5-kbp fragment in the mutant (lane 2). The single hybridization band observed by Southern blotting and the analysis of the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sequence_alignment.png}
\caption{Sequence alignments of the flavoprotein subunit of the SDH complex from \textit{K. lactis} (\textit{KlSDH1}), \textit{S. cerevisiae} (\textit{ScSDH1} and \textit{ScSDH1b}), \textit{H. sapiens} (\textit{SDHA_HUMAN}), and \textit{E. coli} (\textit{SDHA_ECOLI}). The alignments have been performed with MultiAlin (14). Identical amino acids are shaded in black, and isofunctional ones are shaded in gray.}
\end{figure}
entire genome sequences confirmed that K. lactis has a single SDH1 gene (Yvan Zivanovic [Université de Paris-Sud], personal communication). A growth test on minimal media containing various carbon sources confirmed that the deleted strain, like the MS14-1A mutant, was unable to grow on all respiratory carbon sources tested, with the exception of lactate.

We also checked for the presence of SDH activity in both the wild type and in the deletion strains. Mitochondrial and cytoplasmic extracts were prepared from glucose-grown cultures of MW179-1D (lanes 1 and 4), Klsdh1Δ (lanes 2 and 5), and the Klsdh1Δ strain complemented with a centromeric plasmid harboring KISDH1 (lanes 3 and 6). The proteins were separated on native gel and stained for SDH activity as described in Materials and Methods. The arrow indicates the direction of the protein migration.

KISDH1 is not repressed by glucose. The SDH complex of S. cerevisiae has been extensively studied. Like many other genes of the Krebs cycle, the respiratory chain, and gluconeogenesis, SDH1 and SDH2 are regulated at the transcription level by carbon sources (19, 31, 41, 45).

To investigate the regulation of KISDH1, we performed a Northern analysis in three different reference strains of K. lactis, namely CBS2359/152, MW278-20C/1, and MW179-1D. Results showed that, unlike S. cerevisiae, the KISDH1 transcript was always present in all strains grown on both fermentable and nonfermentable carbon sources (Fig. 4). In particular, the amount of the KISDH1 mRNA from ethanol and glycerol cultures was very similar to that of cells grown in 1% glucose, indicating that the gene was not sensitive to carbon catabolite repression. These results are in agreement with the general observation that respiratory genes of K. lactis escape transcriptional repression during growth on glucose (26, 35, 36, 53). No KISDH1 mRNA could be detected in the Klsdh1Δ strain grown on different concentrations of glucose or lactate (lanes 8, 9, and 10).

Growth of the Klsdh1Δ mutant on glucose. We also examined the growth of the mutant on glucose. The wild-type and Klsdh1Δ strains were grown on 50 ml of YP medium containing 1% glucose, and at time intervals, cell numbers, glucose consumption, and ethanol production were determined. Results showed that the Klsdh1Δ mutant had a reduced growth rate both in lag and stationary phases, while the growth curves of the two strains were similar during the exponential phase (Fig. 5A). Despite the small difference between the two strains in biomass yield, the levels of glucose consumption and ethanol production in the mutant cultures were significantly reduced. In fact, after 25 h of growth, about 40% of the glucose was still present in the medium while, in the wild-type cultures, the sugar was completely exhausted (Fig. 5B). Moreover, alcohol production in the mutant cultures was strongly delayed and only after 25 h reached a concentration of about 0.3g/liter, which is about one-seventh the amount produced by the wild type (about 2g/liter). These data clearly indicated that the growth of the Klsdh1Δ mutant was essentially due to the respiration of glucose and not to increased fermentative activity. Interestingly, dry biomass measurements of the same cell concentrations (for instance, 3 × 10^8 cells/ml) showed that the mutant weighs on average 15% less than the wild type. This

![Restriction map of the KISDH1 locus.](image)

**FIG. 3.** (A) Restriction map of the KISDH1 locus. (B) KISDH1 (kanMX4 URA3) deletion cassette. Open bar, open reading frame; black bar, gene promoter and terminator. (C) Southern analysis of the wild-type MW179-1D (1) and Klsdh1Δ isogenic strain (2). Genomic DNAs were digested with SalI and SphI and probed with a PCR-amplified fragment (about 3.6 kbp) containing, besides the coding region, 1.2 kbp of promoter and 0.4 kbp of terminator. (D) Mitochondrial (MIT) and cytoplasmic (CYT) extracts were prepared from glucose-grown cultures of MW179-1D (lanes 1 and 4), Klsdh1Δ (lanes 2 and 5), and the Klsdh1Δ strain complemented with a centromeric plasmid harboring KISDH1 (lanes 3 and 6). The proteins were separated on native gel and stained for SDH activity as described in Materials and Methods. The arrow indicates the direction of the protein migration.

![Transcription analysis of KISDH1 in strain MW179-1D.](image)

**FIG. 4.** Transcription analysis of KISDH1 in strain MW179-1D (lanes 1 to 7) and the Klsdh1Δ (lanes 8 to 10) mutant grown in rich medium (YP) containing 0.3 (D.3), 1 (D1) or 7% (D7) glucose, 2% ethanol (E), 1% succinate (Su), 2% lactate (L), or 2% glycerol (G). Total RNAs were probed with the KISDH1 1.5-kbp BglII-XbaI fragment (represented in Fig. 3A). Expression of the actin gene (KIACT1) was included as a relatively constant reference.
difference could not alone account for the big difference in ethanol production (see below).

**Klsdh1** is dispensable for growth on lactate. In both *S. cerevisiae* and *K. lactis*, DL-lactate is oxidized to pyruvate by the mitochondrial enzymes D-LCR and L-lactate ferredoxin oxidoreductase (L-LCR), encoded by the genes *DLD1* (*Kldld1*) and *CYB2* (*KlcYb2*), respectively, which use cytochrome c as the electron acceptor of the reaction (see Fig. 8) (1, 23, 28, 29). In *S. cerevisiae*, pyruvate derived from lactate is channeled into the Krebs cycle, and the disruption of the *SDH1* gene blocks the capability of cells to utilize all respiratory carbon sources including lactate (Fig. 6, line 2) (27). In contrast, in *K. lactis*, the *Klsdh1*Δ strain, as well as the MS14-1A *sdh* mutant originally isolated, failed to grow on respiratory carbon sources with the exception of lactate (Fig. 6, line 4), indicating that lactate utilization in this yeast can be achieved through an alternative pathway bypassing SDH. Therefore, we analyzed the respiratory capability of the *Klsdh1*Δ strain. Cells grown on YP-glucose medium until the late exponential phase were starved for 24 h previous to the determination. Oxygen consumption was measured in the presence of glucose, ethanol, or lactate. As shown in Table 2, we found that the respiration rate in the presence of glucose was similar or even higher in the mutant compared to the level in the wild type. Moreover, high respiratory activity was also observed in the presence of ethanol, a substrate that the mutant cannot use for growth. On lactate, the mutant showed a rate of respiration that was significant, although reduced to one-eighth of that of the wild type (3.6 instead of 31 μl of oxygen consumed per hour per mg of dry cells). The addition of glucose to the lactate system restored high respiration levels (57.6 μl of oxygen). The addition of antimycin A blocked oxygen consumption completely, consistent with the observation that neither the wild type nor the deletion strain was able to grow on lactate me-

**FIG. 5.** Growth curves of the MW179-1D (wild type or wt) and the Klsdh1Δ (mutant) strain on YP medium containing 1% glucose (A). Glucose (gluc) and ethanol (et) concentrations were determined at time intervals on the culture supernatant (B). Each value in the figures represents the average of three independent determinations. In no case was the variation higher than 15%.

**FIG. 6.** Growth test of the *S. cerevisiae* wild type BY4741 (1), BY4741sdh1Δ (2), and BY4741sdh1Δ (3) strains and *K. lactis* MW179-1D*Klsdh1*Δ (4), MW179-1D (5), GG1993 (*Klpda1*Δ) (6), and CBS2359/152 (7). The plates are minimal media containing glucose or lactate. GG1993 is an isogenic derivative of CBS2359 (50). The initial cell concentration was 2 × 10⁷ to 4 × 10⁷ with a 10-fold dilution.

**TABLE 2.** Respiration rates of strains grown on YP medium containing glucose

| Growth in the presence of: | O₂ consumption (µl of O₂ consumed/h/mg of dry mass)* |
|----------------------------|-------------------------------------------------------|
| Glucose                    | Wild type: 45                                       |
|                            | Klsdh1Δ: 31                                         |
| Lactate                    | Wild type: 31                                       |
|                            | Klsdh1Δ: 3.6 (57.6)                                 |
| Ethanol                    | Wild type: 90                                       |
|                            | Klsdh1Δ: 68.4                                       |

* Strains were grown on YP medium containing glucose until late exponential phase, starved for 24 h, and then shifted on glucose, lactate, and ethanol. All values represent the average of three independent experiments. In no case was the variation greater than 15%.
* Strain MW179-1D.
* The value in parentheses represents oxygen consumption after the addition of glucose to the sample.
dium containing this respiratory chain inhibitor. The oxygen consumption observed on lactate indicated that the SDH-alternative lactate catabolism of Klsth1Δ cells is mediated by the antimycin A-sensitive respiratory chain. Interestingly, as observed in the Klsth1Δ mutant, we found that the K. lactis strain devoid of KIPDAI (54), the gene encoding the E1α subunit of the mitochondrial pyruvate dehydrogenase complex, was able to grow on lactate (Fig. 6, line 6). This suggested that in both mutants, pyruvate might be dissimilated through the pyruvate decarboxylase (PDC) pathway that bypasses the pyruvate dehydrogenase complex.

Expression of pyruvate-related genes in the Klsth1Δ mutant. The Klsth1Δ strain was further analyzed to explain the growth on lactate and the reduced fermentative capabilities. We performed a Northern analysis of the genes involved in pyruvate utilization to look at the metabolic route of its dissimilation. Therefore, we analyzed the expression levels of the genes for pyruvate decarboxylase (KIPDCA) (6) and acetyl-coenzyme A (acetyl-CoA) synthetase (KlACS1 and KlACS2) (55), and two genes of the glyoxylate cycle, namely isocitrate lyase (KlICL1) (32) and malate synthase (KlMLS1) (see Fig. 8 for a representation of the reactions catalyzed by these activities). Wild-type and Klsth1Δ mutant strains were grown overnight on YP medium containing 1% glucose or 2% lactate, and total RNAs were prepared from these cultures. In K. lactis the regulation of the two genes for acetyl-CoA synthetase has been previously described: KlACS1 is expressed at low level on glucose or ethanol and induced on acetate or lactate, while KlACS2 is preferentially expressed on glucose and ethanol (30, 55). Such regulation of the ACS genes is present also in our wild-type strain grown on glucose (Fig. 7 lane 1) and lactate (lane 3). Interestingly, KlACS1 and KlACS2 in the mutant strain were expressed at higher levels compared to the wild type when the mutant strain was grown in lactate (lane 4) and glucose (lane 2), respectively. Compared to expression in the wild-type strain (lanes 1 and 3), increased expression of these genes in the mutant (lanes 2 and 4) was also observed for KlMLS1 and KlICL1 on both glucose and lactate. While the low level of the KIPDCA transcript observed in the Klsth1Δ mutant on glucose (lane 2) was unexpected since this gene is normally induced by this carbon source (6), the level of expression is in agreement with the reduced fermentative capabilities of the mutant. The high level of expression of KIPDCA on lactate in both strains (lanes 3 and 4) confirmed that this gene could play a role in lactate utilization.

Succinate determination in the Klsth1Δ mutant. The activation of the glyoxylate cycle, indicated by the high levels of the KlICL1 and KlMLS1 transcripts (Fig. 7, lanes 2 and 4), should follow the deletion of KlSDH1. If the activation of this cycle occurred, we would expect from one mole of glucose the production by isocitrate lyase of one mole of glyoxylate and one mole of succinate. Figure 8 shows a schematic representation of the metabolic routes of the Krebs and the glyoxylate cycles that determine succinate utilization and production. The inability of the Klsth1Δ mutant to oxidize succinate would lead to succinate accumulation into the medium. To test this hypothesis, the wild type and Klsth1Δ strain were grown on YP medium containing 1% glucose or 2% lactate, and the production of succinate was determined at 24 and 36 h. Indeed, the Klsth1Δ mutant, when grown on glucose, accumulates succinate at concentrations of about 0.5 g/liter at 24 h and 1.2 g/liter at 36 h. In contrast, the amount of succinate in the wild type was, under the same conditions, below the sensitivity of the system. No succinate was detected in either the wild-type or mutant strain during growth on lactate.

DISCUSSION

Glucose metabolism in the Klsth1Δ mutant. SDH is an important complex of the Krebs cycle that catalyzes the oxidation of succinate to fumarate and feeds electrons to the respiratory chain ubiquinone pool. SDH1 is the catalytic component of this complex. While S. cerevisiae has two paralogues of SDH1, KlSDH1 appears to be a unique gene in K. lactis. More important, the regulation of the expression of these genes is very different in the two yeasts. In fact, in S. cerevisiae SDH1 is derepressed at the transcriptional level in the presence of non-fermentable carbon sources, while in K. lactis, KlSDH1 is expressed even in glucose-grown cells. S. cerevisiae strains devoid of SDH1 were unable to grow on respiratory carbon sources, whereas a Klsth1 null strain was still able to grow on lactate. Moreover, the Klsth1Δ mutant showed decreased glucose consumption and severely reduced ethanol production. Accordingly, we found a reduced level of expression of the pyruvate decarboxylase gene (KIPDCA) during growth on glucose. Recently, it has been reported that KlICL1, the K. lactis gene coding for isocitrate lyase, a key enzyme of the glyoxylate cycle,
is glucose repressed (32). On the contrary, in the Klsdh1Δ mutant we found not only specific induction of this gene but also increased expression of the malate synthase (KlMLS1) and acetyl-CoA synthetase (KlACS2) genes, which suggested a re-routing of the glucose flux towards the glyoxylate cycle. It follows that acetaldehyde, due to the inability of the Klsdh1Δ mutant to accumulate ethanol, is converted to acetate activated to acetyl-CoA that is finally channeled into the glyoxylate cycle. The use of this cycle in a strain blocked in SDH would lead to the accumulation of succinate, as demonstrated in the scheme of Fig. 8 that shows the metabolic pathways involved in the production and utilization of this organic acid. This hypothesis was confirmed in the Klsdh1Δ mutant by the accumulation of succinate (1.2g/liter at 36 h). Therefore, the accumulation of succinate instead of ethanol in K. lactis suggests a bond between the expression of the KlSDH1 gene on glucose and efficient fermentation. The production of succinate was previously observed to a lower extent under very different growth conditions (15% glucose) also on the S. cerevisiae sdh1/H9004 mutant (2). According to the authors of that study, in this yeast succinate accumulation was not dependent on the glyoxylate cycle but on the activation of the oxidative part of the Krebs cycle. In both cases, the redox potential may be directly transferred via mitochondrial membrane dehydrogenases (3, 37) to the ubiquinone pool, thus avoiding the SDH block in the Krebs cycle (8).

How can the Klsdh1Δ mutant grow on lactate and not on other respiratory substrates? K. lactis cells lacking SDH showed a high respiration rate on ethanol but failed to grow on this carbon source as well as on pyruvate and glycerol, substrates that in principle can be dissimilated through the PDC pathway. This fact indicates that respiration is a condition necessary but not sufficient for the utilization of nonfermentable carbon sources. One possible explanation is that, following the impairment of the Krebs cycle, the synthesis of ATP

FIG. 8. Schematic drawing of the hypothetical metabolic routes (glycolysis, Krebs, and glyoxylate cycles), modified from Tabak et al. (46), involved in succinate accumulation in the Klsdh1Δ mutant. The major sources of NAD(P)H production during growth on glucose and lactate are indicated. The pentose phosphate shunt has been included because in K. lactis this pathway is probably constitutively operative. The NADPH produced in this pathway can be reoxidized directly via mitochondrial membrane dehydrogenase; ACS, PDC, MLS, and ICL indicate the genes coding for the following activities, respectively: acetyl-coA synthetase, pyruvate decarboxylase, malate synthase, and isocitrate lyase. LDH indicates the mitochondrial activities responsible for lactate oxidation encoded by the KlCYB2 and KlLD1 genes. PDH, pyruvate dehydrogenase complex. To indicate the block in the SDH route, SDH has been crossed out (×).
required for acetate activation becomes the limiting step for the utilization of these substrates. In the case of lactate, the fundamental step for the utilization of this substrate is its oxidation to pyruvate. This reaction catalyzed by the mitochondrial D-LCR and L-LCR use cytochrome $c$ as the electron acceptor of the reaction. Reoxidation of cytochrome $c$ at the level of complex IV may produce the proton force gradient for ATP synthesis necessary for pyruvate activation. Since we have found that the \textit{Klpda1} strain is able to grow on lactate (Fig. 6, line 6), lactate-derived pyruvate must be utilized through the pyruvate decarboxylase pathway. In fact, the high transcription levels of the \textit{KIPDCA}, \textit{KIACS1}, \textit{KICL1}, and \textit{KIMLS1} genes suggest activation of this pathway that leads to the glyoxylate cycle, as shown in the scheme of Fig. 8. Although this metabolic route is required in the \textit{K. lactis} \textit{Klsdh1}\textDelta mutant for growth on lactate, it is not clear how this mutant avoids succinate accumulation. We can hypothesize that, on lactate, the request of intermediates necessary for the synthesis of heme, gluconegenesis, or other metabolites is so high that succinate accumulation is prevented. Alternatively, we could think that other activities, not expressed on glucose and specific to other pathways, are activated leading to the glyoxylate cycle as shown in the scheme of Fig. 8. Therefore, in order to unveil the metabolic route(s) beyond lactate utilization. The reduced oxygen consumption of the \textit{Klsdh1}\textDelta mutant cells on lactate, compared to consumption in the wild type, could be due to the low levels of the reducing equivalents produced mainly in the conversion from acetaldelyde to acetate and from malate to oxaloacetate (Fig. 8). We also asked why \textit{S. cerevisiae} \textit{sdh1} mutants that harbor mitochondrial D-LCR and L-LCR are unable to grow on lactate. One possible explanation is that respiration, inferred by many authors only by nongrowth on respiratory carbon sources, and/or the PDC pathways are not active in such mutants. In conclusion, the reported pathway for lactate utilization could be a peculiar trait of \textit{K. lactis}, acquired in the milk-derived niches that are the natural habitats of this yeast (1, 48). We hypothesize that the evolution of this SDH-independent lactate pathway in \textit{K. lactis} might have evolved from the coexistence in the same habitat of lactate-producing bacteria and microaerobic conditions. The idea that \textit{K. lactis} could choose the modality of lactate utilization depending on oxygen availability is appealing, and we wonder if a similar mechanism may exist also in higher eukaryotes.

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