Improved bioethanol production in an engineered *Kluyveromyces lactis* strain shifted from respiratory to fermentative metabolism by deletion of NDI1

María Isabel González-Siso,† Alba Touriño, Ángel Vizoso, Ángel Pereira-Rodríguez,‡ Esther Rodríguez-Belmonte, Manuel Becerra and María Esperanza Cerdán
Grupo de Investigación EXPRELA, Departamento de Biología Celular e Molecular, Facultade de Ciencias, Universidade da Coruña, Campus de A Coruña, 15071-A Coruña, Spain.

**Summary**

In this paper, we report the metabolic engineering of the respiratory yeast *Kluyveromyces lactis* by construction and characterization of a null mutant (Δklndi1) in the single gene encoding a mitochondrial alternative internal dehydrogenase. Isolated mitochondria of the Δklndi1 mutant show unaffected rate of oxidation of exogenous NADH, but no oxidation of matrix NADH; this confirms that K/NDi1p is the only internal NADH dehydrogenase in *K. lactis* mitochondria. Permeabilized cells of the Δklndi1 mutant do not show oxidation of matrix NADH, which suggests that shuttle systems to transfer the NADH from mitochondrial matrix to cytosol, for being oxidized by external dehydrogenases, are not functional. The Δklndi1 mutation decreases the chronological life span in absence of nutrients. The expression of KNDI1 is increased by glutathione reductase depletion. The Δklndi1 mutation shifts the *K. lactis* metabolism from respiratory to fermentative: the Δklndi1 strain shows reduced respiration rate and increased ethanol production from glucose, while it does not grow in non-fermentable carbon sources such as lactate. The biotechnological benefit of the Δklndi1 mutant for bioethanol production from waste cheese whey lactose was proved.

**Introduction**

In contrast to mammals, but similar to other eukaryotes like filamentous fungi or plants, in the inner mitochondrial membrane of yeasts, there are the so-called alternative dehydrogenases, or class-2 dehydrogenases, which are able to oxidize cytosolic and mitochondrial matrix NAD(P)H coenzymes and to transfer the electrons to ubiquinone. As opposed to complex I, these are non-proton-pumping, rotenone-insensitive and single polypeptide enzymes. Their precise physiological role in the different organisms has not been completely established, although it is known that they constitute a main entry point for electrons in the mitochondrial respiratory chain, being involved in the regulation of intracellular redox balance and energy production (Tarrío et al., 2006b). The characteristics of the mitochondrial alternative NAD(P)H dehydrogenases studied hitherto in yeasts are related to the preponderance of respiratory or fermentative metabolism in different species (González-Siso and Cerdán, 2007).

*Saccharomyces cerevisiae* and *Kluyveromyces lactis* are two yeasts that metabolize the glucose by oxidative and fermentative pathways, but *S. cerevisiae* is predominantly fermentative (Crabtree positive) and *K. lactis* is predominantly respiratory (Crabtree negative) (González-Siso et al., 2000). Both species lack complex I, which is present in strictly aerobic yeasts, and contain one internal alternative dehydrogenase and two external alternative dehydrogenases (Tarrío et al., 2006a; González-Siso and Cerdán, 2007).

The external alternative dehydrogenases show differential characteristics between the fermentative and the respiratory yeast. In *S. cerevisiae*, a decrease in expression of the external NADH dehydrogenases is observed at high glucose concentrations and this feature has been related to a higher amount of reoxidation of cytosolic NADH by the alcohol dehydrogenases and therefore with the prevalence of aerobic fermentation (the Crabtree-positive phenotype) (Luttik et al., 1998). In *K. lactis*, the ability of the external dehydrogenases to use NADPH, in addition to NADH, supports an increased use of the pentose phosphate pathway (PPP) to metabolize glucose in detriment of glycolysis (González-Siso et al., 1996a,b; Tarrío et al., 2005; 2006b).
Differently to mitochondria from plants and filamentous fungi, where complex I and internal alternative dehydrogenase coexist (MOller, 2002; Duarte and Videira, 2007), in S. cerevisiae and K. lactis, complex I is replaced by the alternative enzyme. Therefore, in these yeasts, the internal dehydrogenase constitutes the metabolic equivalent of complex I. In accordance with this function and in both yeasts, the expression of the alternative internal dehydrogenase is reduced at high glucose concentrations when fermentation is favoured (De Risi et al., 1997; Tarrío et al., 2005).

The ability to complement complex I defects has been proved for the S. cerevisiae alternative internal dehydrogenase, ScNdi1p (Yagi et al., 2006; Marella et al., 2008). Deficiency of complex I is one of the most frequent dysfunctions of the mitochondrial respiratory chain in human, often encompassing a large spectrum of clinical presentations, and is closely related to the aetiology of sporadic Parkinson’s disease (Marella et al., 2008) and ageing (Balaban et al., 2005). The potential of the yeast NDI1 gene in therapy of complex I defects has been demonstrated in rat animal models (Marella et al., 2008; 2010). This potential might be limited by the difference between the fermentative metabolism shown by S. cerevisiae cells and the oxidative metabolism shown by most tissues affected by complex I deficiency like nervous and cardiac; therefore, the use of the NDI1 genes from Crabtree-negative yeasts could become advantageous (González-Siso et al., 2009).

In S. cerevisiae, ScNdi1p is also the closest homologue of mammalian AMID (apoptosis-inducing factor-homologous mitochondrion-associated inducer of death). Glucose repression of respiration (and also of antioxidant defences like the mitochondrial superoxide dismutase) has been proposed to be linked to the cell death process in yeast (Li et al., 2006). Besides, mitochondrial release (Cui et al., 2012) or overexpression of ScNdi1p (Li et al., 2006) has been directly related to apoptosis in S. cerevisiae.

In contraposition to S. cerevisiae, K. lactis shows an oxidative metabolism, is scarcely sensitive to glucose repression of respiration (González-Siso et al., 2000) and does not present an increase in longevity or a decrease in reactive oxygen species (ROS) formation when cultivated in lower glucose concentrations (Oliveira et al., 2008). These differences might involve the specific characteristics of the NDI1 genes and their products of both yeasts, and their knowledge is important for their use in gene therapy of complex I disorders. Moreover, the regulation of NDI1 genes is an important point to control the respiro-fermentative metabolism of yeasts and consequently their biotechnological use as cell factories (González-Siso et al., 2009) for recombinant protein production (González-Siso et al., 2008) or other productions, like bioethanol.

KIND1 deletion shifts metabolism to fermentative

The above exposed reasons led us to study KlNdi1p, the alternative internal dehydrogenase of K. lactis mitochondria. In this work, we have constructed a null mutant in KIND1, the gene encoding this enzyme and used it to perform a functional analysis of KlNdi1p. The specific characteristics found in the null strain made it valuable to produce bioethanol from cheese whey lactose, a waste product, thus also proved in this work.

Results and discussion

Construction of the Klndi1 null mutant

The KIND1 gene was isolated from a K. lactis genomic DNA library as previously described (Tarrío et al., 2005) and subcloned in a commercial S. cerevisiae episomal vector. The resulting plasmid was used for the construction of the deletion cassette in which the coding region of KIND1 was replaced by the KanMX4 module. The deletion cassette was introduced into the PM5-3C strain and the transformants selected for G418 (geneticin) resistance. Analytical polymerase chain reaction (PCR) confirmed the correct integration of the cassette in one of the two G418-resistant transformants obtained, which was named Δklndi1 (Fig. 1) and used in the following analyses.

Isolated mitochondria of the Klndi1 null mutant show unaffected rate of oxidation of exogenous NADH but no oxidation of matrix NADH

Mitochondria from the Δklndi1 mutant and from the corresponding wild-type PM5-3C strain were isolated and used to measure oxygen consumption as described in Experimental procedures. Both externally added NADH and a combination of malate and pyruvate were used as substrates. The combination of malate and pyruvate produces NADH in the mitochondrial matrix via the tricarboxylic acid cycle. Externally added NADH cannot pass through the membrane and enter the mitochondrial matrix; it can be oxidized by external alternative dehydrogenases in intact isolated yeast mitochondria.

Oxygen consumption by isolated mitochondria from the Δklndi1 mutant compared with the wild-type strain (Fig. 2) shows that oxidation of matrix NADH is impaired in the mutant whereas oxidation of externally added NADH remains equal. This confirms that KlNdi1p, depleted in the mutant, is the single internal mitochondrial NADH : ubiquinone oxidoreductase in the mitochondria of K. lactis, and that activity of external alternative dehydrogenases is not affected by the Δklndi1 mutation. A similar phenotype has been reported for isolated mitochondria from the S. cerevisiae Δndi1 mutant (Marres et al., 1991; Bakker et al., 2000).
In the Klnd1 null mutant, there is no overexpression of the external alternative dehydrogenases

Analysis of the expression of the three K. lactis mitochondrial alternative dehydrogenases in the Δklnd1 mutant compared with the wild-type strain was done (Fig. 3). In the Δklnd1 mutant, the band corresponding to the expression of the KlNDI1 gene disappears as expected and quantification of the bands corresponding to KlNDE1 and KlNDE2 expression, responsible of cytosolic NADH oxidation, shows no statistically significant differences in reference to those in the wild-type strain (P values of 0.99 and 0.11, respectively, in the t-test performed to compare the means). This is in accordance with the above exposed results measuring oxygen consumption using NADH form cytoplasmic or matrix origin (Fig. 2) and corroborates that either expression or activity of external alternative dehydrogenases is not affected in the Δklnd1 strain.

The growth of the Klnd1 null mutant in glucose is unaffected and in lactate is impaired

To test the effect of KlNDI1 disruption on K. lactis metabolism, we examined the growth of the Δklnd1 mutant in comparison with that of the parental PM5-3C strain in liquid (shake flask cultures) and solid complete medium (CM) containing various carbon sources under the conditions described in Experimental procedures. The mutant was no longer able to utilize non-fermentable substrates such as lactate while showed the same growth rate than the
parental strain in fermentable substrates such as glucose (Fig. 4). A similar phenotype was reported for the S. cerevisiae Δndi1 mutant (Marres et al., 1991; Bakker et al., 2001; Li et al., 2006). Also Δndi1 mutants of both K. lactis (this work) and S. cerevisiae (Marres et al., 1991; Bakker et al., 2001) exhibited similar growth rate as wild-type cells in galactose. However, a difference is that ethanol sustained a more limited growth of the Δklndi1 mutant than the wild type (Fig. 4), while growth of the S. cerevisiae Δndi1 mutant in ethanol was unaffected with respect to the wild type (Marres et al., 1991; Bakker et al., 2001).

For growth on lactate and ethanol in the Δklndi1 mutant, with the NAD⁺-linked steps of the Krebs cycle inhibited, the glyoxylate cycle and glucogenogenesis pathways have to be induced. The amount of ATP generated in this metabolic condition is lower than when the Krebs cycle and respiratory chain are fully operative as occurs in the wild-type strain, which may explain the reduced growth in non-fermentable carbon sources of the Δklndi1 strain (Marres et al., 1991). The better growth of the Δklndi1 strain observed in ethanol than lactate may also be explained by energetic and redox balances. For lactate utilization, three genes have been characterized so far in K. lactis, namely KUEN1, KIDL and KICYB2, which encode a permease and two lactate ferricytochrome c oxidoreductases respectively (Rodicio and Heinisch, 2013). Lactate dissimilation by S. cerevisiae is also initiated by the oxidation of lactate to pyruvate via a cytochrome c-dependent mitochondrial lactate dehydrogenase and does not yield cytosolic NADH, while ethanol dissimilation by cytosolic alcohol dehydrogenases yields NADH that can be oxidized by the respiratory chain via both external mitochondrial dehydrogenases and the glycerol 3-phosphate shuttle, consisting of cytosolic NADH-linked glycerol 3-phosphate dehydrogenase and a membrane-bound FAD⁺-linked glycerol 3-phosphate : ubiquinone oxidoreductase (Bakker et al., 2001). Both cytosolic NADH-oxidation systems are in concerted regulation (Rigoulet et al., 2004). In K. lactis, two cytosolic isoforms of alcohol dehydrogenase, encoded by KIAH1 and KIAH2, exist and these two mechanisms for cytosolic NADH oxidation and ATP generation in mitochondrial respiratory chain are also operative (González-Siso and Cerdán, 2007; Saliola et al., 2010), as schematized in Fig. 5A. These metabolic characteristics may explain the growth of the Δklndi1 mutant in ethanol, limited but at a higher rate than in lactate.

Permeabilized cells of the Klndi1 null mutant show no oxidation of mitochondrial matrix NADH

In S. cerevisiae, there is experimental evidence that another important component of the redox-balancing
system is the ethanol/acetaldehyde (Et–Ac) shuttle (Bakker et al., 2001). These metabolites, which can freely diffuse through mitochondrial membranes, are oxidized or reduced by cytosolic or mitochondrial alcohol dehydrogenases transferring the NADH excess from one cellular compartment to the other. The Et–Ac shuttle allows respiratory growth of the \( S.\) \( cerevisiae \) \( \Delta ndi1 \) mutant at the same rate than the wild-type strain in both glucose and ethanol as carbon sources. This is because matrix NADH is exchanged by the reversible interconversion of acetaldehyde into ethanol to the cytosol, where it can be oxidized by external mitochondrial dehydrogenases and the glycerol 3-phosphate shuttle.

Nevertheless, this system is limited, since the mutant does not grow on acetate (Bakker et al., 2001). Saliola and colleagues (2010), based only on the fact that the addition of small amounts of ethanol, unable to sustain growth by itself, allowed a slight growth recovery in lactate medium of a \( \Delta ndi1 \) mutant, have proposed that in \( K.\) \( lactis \) the Et–Ac shuttle might be operative and could transfer the redox excess from the mitochondria to the cytoplasm, thus feeding the respiratory chain through the external dehydrogenases \( K\)\(Nde1p \) and \( K\)\(Nde2p \) (Fig. 5A).

To verify if the Et–Ac shuttle is active in \( K.\) \( lactis \) and, in complete absence of \( KNDI1 \) expression, might exchange matrix NADH to be oxidized by cytosolic systems, we measured oxygen consumption by permeabilized cells of the \( \Delta kndi1 \) mutant with 0.1 M ethanol as substrate instead of isolated mitochondria allows to test reoxidation of internal produced NADH in conditions in which the cytoplasmic component of the shuttle is active. Blockage of oxygen consumption by antimycin A in \( \Delta kndi1 \)-permeabilized cells proved that measured respiration rate corresponds to the mitochondrial respiratory chain (Fig. 5B). However, permeabilized cells of the \( \Delta kndi1 \) mutant do not consume oxygen in the presence of the combination of pyruvate and malate as substrate (Fig. 5C). As a control, we measured oxygen consumption with ethanol as substrate and there is no significant difference between permeabilized cells of the \( \Delta kndi1 \) mutant in comparison to the wild-type strain (Fig. 5C). This may be because ethanol metabolism produces

---

**Fig. 5.** (A) Scheme of the \( K.\) \( lactis \) electron transport chain and cytosol-mitochondria NADH shuttle systems. Adh, alcohol dehydrogenases; Ndi1, internal NADH dehydrogenase; Nde1/2 external NAD(P)H dehydrogenases; Q, ubiquinone; DHAP, dihydroxy acetone phosphate; G3P, glycerol 3-phosphate; Gpd, glycerol 3-phosphate dehydrogenase; Gut2, FAD-linked glycerol 3-phosphate dehydrogenase; cIII, complex III; cIV, complex IV. (B) Oxygen uptake by permeabilized cells of the \( \Delta kndi1 \) mutant with 0.1 M ethanol as substrate stops upon antimycin A addition. (C) Respiration rate (nmoles \( O_2/\)min \( \cdot ml^{-1} \)) by permeabilized cells of the \( \Delta kndi1 \) mutant compared with the PM5-3c strain (wild type for \( NDI1 \)) with the substrates pyruvate + malate and ethanol. Results are the mean ± standard deviation (SD) of three independent experiments. **\( P < 0.01.\)
NADH in the mitochondrial matrix but also in the cytosol since K. lactis has mitochondrial and cytosolic alcohol dehydrogenases and a functional glyoxylate cycle (Rodicio and Heinisch, 2013), and cytosolic NADH can be oxidized by the external alternative dehydrogenases of the respiratory chain. Therefore, the Et–Ac shuttle seems to be inactive in the Δklglr1 mutant cells, at least in the conditions assayed.

The expression of the KINDI1 gene is regulated by KIGLR1 function

In previous papers, we proved the involvement of KIGlr1p, the K. lactis NADPH-dependent glutathione reductase (GLR), not only in the defence against oxidative stress but also in the regulation of the glucose metabolism in K. lactis. This second role was inferred both from the improved growth on glucose caused by the Δklglr1 mutation and by the increased GLR activity in the Δklgrcr1 strain (García-Leiro et al., 2010a) being KIGcr1p a positive transcriptional regulator of glycolytic genes, as well as by the fact that enzymatic activities of GLR and glucose 6-phosphate dehydrogenase (G6PDH) from the PPP are positively correlated (Tarrío et al., 2008). Thus, G6PDH is positively regulated by an active respiratory chain and GLR plays a role in the reoxidation of the NADPH from the PPP in these conditions (Tarrío et al., 2008). A further proteomic analysis provided additional results; H2O2 addition causes downregulation of enzymes from the glycolytic pathway and Krebs cycle in wild-type K. lactis, whereas Δklglr1 deletion prevents this effect and actually upregulates glycolytic, Krebs cycle and oxidative PPPs, which led us to propose that the redox imbalance produced by the Δklglr1 deletion controls glucose metabolic fluxes in K. lactis (García-Leiro et al., 2010b). New data obtained in this work also support this proposal (Fig. 6). Thus, RNA was extracted from cells grown in CM with 2% glucose (OD600 = 0.8) and KINDI1 mRNA levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in a Δklglr1 mutant (Garcia-Leiro et al., 2010a) and the isogenic parental strain. Significant differences were found: KINDI1 mRNA levels being 1.58-fold higher in the Δklglr1 mutant. The interpretation of these results is that GLR depletion, by reducing NADPH oxidation rate and therefore NADP+ availability, necessary for G6PDH activity, could redirect glucose flow from the PPP which is preponderant in K. lactis for glucose metabolism (González-Siso et al., 2000; 2009) to glycolysis and Krebs cycle, which will be associated to increased KINDI1 expression.

The chronological life span of the Klndi1 null mutant is decreased

It has been reported that ScNdi1p is the closest homologue of mammalian AMID in yeast and is involved in chronological ageing (Li et al., 2006). Overexpression of ScNdi1p causes apoptosis-like cell death. This effect can be repressed by increased respiration on glucose-limited media, being the apoptotic effect of ScNDI1 overexpression associated with increased production of ROS in mitochondria (Li et al., 2006). We have determined that also in K. lactis, the closest homologue to human AMID is KlNdi1p (González-Siso and Cerdán, 2007), and therefore studied its putative role in ageing as following described.

Chronological life span of the Δklndi1 mutant and parental strains was measured using the protocols described in the Experimental procedures. For cell grown in a CM and further maintained in distilled water, significant differences in viability (quantified as half-life) were found between the Δklndi1 mutant and wild-type strain (Fig. 7A), the viability being decreased in the mutant. This situation is different to the one reported in S. cerevisiae where disruption of NDI1 decreases ROS production and elongates the chronological life span of yeast, accompanied by the loss of survival fitness (Li et al., 2006). However, considering that these authors measured life span with culture medium renewal each 3 days, while our conditions are survival in absence of nutrients (distilled water), we also measured the life span of the Δklndi1 mutant and parental strains using the same protocol than Li and colleagues (2006) without finding increased life span in the mutant (Fig. 7B). In the latter conditions but before medium renewal, there was neither significant difference in ROS levels (in exponential and stationary culture phase, measured as in Tarrío et al., 2008), nor DNA and nuclear fragmentation between the Δklndi1 mutant and wild-type strain (data not shown). The differences caused by NDI1 deletion in the life span of the two species might be related to differences in
respiratory metabolism and ROS production (reviewed in González-Siso et al., 2009), and their dependence on NDI1 function, but further work is necessary to clarify this issue.

Interestingly, the Δnde1 mutation has been reported to shorten replicative life span in S. cerevisiae and the authors proposed that this effect results from multiple mechanisms including the low respiration rate and low energy production rather than just from a ROS-dependent pathway; however, regarding chronological life span, measured in rich medium, Δnde1 cells were able to survive better than wild-type cells in the stationary phase (Hacioglu et al., 2012).

The Klndi1 null mutant shows overall reduced respiration rate and increased ethanol production from glucose

To increase supporting evidence of the effect of KINDI1 disruption on yeast metabolism, we measured the overall respiration rate, with glucose and ethanol as substrates, in cells of the Δklndi1 mutant and parental strains, sampled at two OD600 values (2 and 6) from glucose (YPD) cultures. Figure 8 shows that overall respiration rate of the mutant compared with the parental strain is reduced both with glucose or ethanol as substrates. There is also a significant difference in cell respiration rate of the Δklndi1 mutant (but not in the case of the wild-type strain) being lower in ethanol compared with glucose as substrate.

We also measured ethanol production in glucose liquid cultures of the Δklndi1 mutant and wild-type strains, obtaining increased ethanol production in the mutant. Thus, after 2 days of culture in CM with 2% glucose, when glucose concentration in the medium was lower than 1 g l−1 and OD600 about 10, ethanol in the culture medium was higher for the mutant strain than for the PM5-3C strain (1 ± 0.18 g l−1 versus 3.3 ± 0.17 g l−1, n = 2). Similar results were obtained in a Δklndi1 mutant obtained in a different genetic background (Saliola et al., 2010). Higher ethanol levels are due to the lower respiration rate of the mutant (Fig. 8) and, moreover, to ethanol accumulation due to its limited use as carbon source in the Δklndi1 mutant, also proved in this work (Fig. 4).

Both respiration rate and ethanol production measurements indicate a more fermentative glucose metabolism in the case of the Δklndi1 mutant compared with the parental strain that has biotechnological application in whey utilization as following reported.

Ethanol production from cheese whey lactose by the Klndi1 null mutant

Actual market trends point to a worldwide gradual increase in cheese production that generates whey as waste product (9 kg of whey per 1 kg of cheese). Whey disposal constitutes a pollution problem even though systems for its treatment have been developed. The most abundant component of cheese whey is the disaccharide lactose that remains in the permeate after whey ultrafiltration for protein recovery. The treatment of whey permeate by fermenting lactose to ethanol has received wide attention to date and there are industrial plants in...
operation (Guimarães et al., 2010). However, the development of a high productivity lactose fermenting process continues to be of prime importance. *Saccharomyces cerevisiae* is unable to ferment directly lactose to ethanol and the microorganisms mainly used for this purpose are selected *Kluyveromyces* spp. To reduce distillation costs, it is recommended to use concentrated whey up to 120 g lactose l$^{-1}$ (González-Siso, 1996; Arif et al., 2008).

In this work, we took advantage of the predominantly fermentative metabolism shown by the Δklnidi1 mutant and assayed the use of this strain for the bioconversion of lactose to ethanol in concentrated cheese whey permeate. Figure 9 shows that the Δklnidi1 mutant, grown in the fermenter culture conditions described in Experimental procedures, fully degrades the lactose in 8–9 days and produces up to 50 g l$^{-1}$ ethanol which gives a yield (80.1% of the theoretical maximum of 4 mol of ethanol from 1 mol of lactose) close to the maximum reported for industrial strains (Della-Bianca et al., 2013; Diniz et al., 2013), and higher than the NRRL-Y1140 laboratory strain that is wild type for the *KINDI1* gene (the yield ranged between 62.6% and 50% of the theoretical maximum in the same conditions, according to the highest value and the value adjusted to the curve respectively). Although the yield obtained with the NRRL-Y1140 strain seems high for a respiratory yeast, the distribution between respiratory and fermentative metabolism in wild-type *K. lactis* is dependent on oxygen availability (González-Siso et al., 1996a; Guimarães et al., 2010) and low levels of oxygen in these cultures favour fermentation. Similar ethanol yields in whey than those reached by the NRRL-Y1140 strain in this work were obtained by other authors in fermentative conditions with other *Kluyveromyces* strains (Szczodrak et al., 1997; Guimarães et al., 2010).

Once we proved the ethanol production performance of the Δklnidi1 mutant, we decided to transform this strain with the multicopy plasmid pSPGK1-LAC4 (Becerra et al., 2001) to improve its capability to grow in lactose and therefore ethanol production. The pSPGK1-LAC4 plasmid expresses the LAC4 gene that codes for *K. lactis* beta-galactosidase. The results show that the Δklnidi1 mutant, in the sealed tube conditions described in Experimental procedures, degrades the 79% of the lactose in 4 days (a 56% more than the untransformed strain) producing ethanol with a yield (quantified as percentage of the theoretical maximum of 4 mol of ethanol from 1 mol of lactose) 16.4% higher for the transformed versus untransformed strain. Overexpression of beta-galactosidase increases both lactose consumption rate and yield of ethanol from lactose.

These results support the biotechnological use of Δklnidi1 and its derivative pSPGK1-LAC4 transformants for efficient fermentation of cheese whey to bioethanol.

**Conclusions**

In this paper, we have constructed and characterized an engineered *K. lactis* strain by deletion of the *KINDI1* gene. We proved that *KINDI1* encodes the single internal NADH : ubiquinone oxidoreductase in the mitochondria of *K. lactis* (KlNdi1p), and that activity of external alternative dehydrogenases is not affected by the Δklnidi1 mutation. Similarly to data previously reported for the null in *S. cerevisiae*, the Δklnidi1 mutant grows at the same rate
than the wild-type strain in glucose but is unable to grow in lactate. Differently to the phenotypes reported for the \(\Delta ndi1\) in \(S.\) \(cerevisiae\), the \(\Delta klndi\) strain grows in ethanol with a lower rate and shows reduced chronological life span in absence of nutrients compared with the wild-type strain. Besides, shuttle systems transferring NADH from the mitochondrial matrix to the cytosol for oxidation by external dehydrogenases do not operate in permeabilized \(K.\) \(lactis\) cells. As a result of multiple functional differences, the \(\Delta klndi\) mutation shifts glucose metabolism to fermentative, thus increasing the performance of the \(\Delta klndi\) strain for bioethanol production from lactose present in cheese whey. This is a biotechnological advantage versus \(S.\) \(cerevisiae\), which is unable to metabolize lactose, and versus \(K.\) \(lactis\) expressing \(NDI1\), which is highly respiratory. The \(\Delta klndi\) strain combines three important characteristics: the ability to use lactose, high levels of ethanol production and low use of the ethanol produced as carbon source.

**Experimental procedures**

**Strains, media and culture conditions**

The \(K.\) \(lactis\) strain PM5-3C (\(MATa uraA Rag\)) (Wésolesowski-Louvel et al., 1992) was used.

Growth and handling of yeasts were carried out according to standard procedures (Kaiser et al., 1994). The yeast cells were cultivated, unless otherwise stated, in Erlenmeyer flasks at 30°C and 150–250 r.p.m. in the synthetic CM (Zitomer and Hall, 1976), the dropout medium CM-ura (without uracil) or YP medium (2% bactopeptone, 1% yeast extract) containing one of the following carbon sources: 0.1–2% glucose, 2% lactate, 2% ethanol and 2% galactose. Lactate and ethanol media were supplemented with 0.05% glucose. The flasks were filled with 40% volume of culture medium. Solid growth media also contained 1.5% agar.

For growth analysis on solid CM, cells from overnight Erlenmeyer cultures in the same medium were diluted to \(OD_{600} = 0.1\) and then several serial 1:10 dilutions were made. Drops (0.005 ml) of the dilutions were added to the solid media, incubated at 30°C and photographed after 1.5 and 3 days.

**Construction of null mutant**

Deletion of nucleotides +21 to +1523 in the \(KINDI1\) gene (Tarrio et al., 2005) was carried out by the one-step method (Rothstein, 1991). The template of the deletion cassette was constructed as described in Zaragoza (2003) in the plasmid YEplac195, and included the kanamycin resistance gene (the \(kanMX4\) module) flanked by 420 bp homologous to the \(KINDI1\) 5’ region and 569 bp homologous to the \(KINDI1\) 3’ region. The deletion cassette was PCR amplified and the product transformed to the \(K.\) \(lactis\) wild-type strain PM5-3C. For selection of transformants, geneticin was added at a final concentration of 0.3 mg ml\(^{-1}\) to YPD medium. Correct \(KINDI1\) gene deletion was verified by analytical PCR. Genomic DNA as template and several combinations of inward primers, binding outside the deletion cassette, and outward primers, binding within the deleted region, were used. Also, the absence of \(KINDI1\) mRNA in the null mutant was verified by reverse transcriptase-polymerase chain reaction (RT-PCR).

**Isolation of RNA, production of cDNA and RT-PCR**

The kits NucleoSpin (Macherey-Nagel) and SuperScript II (Promega) were used for RNA extraction from the \(K.\) \(lactis\) cells and for cDNA synthesis and RT-PCR, respectively, following the instructions of the suppliers. Twenty cycles of PCR were performed with primers, which amplified the cDNA from positions +1020 to +1380 of the \(KINDI1\) cds, +1088 to +1603 of the \(KINDE2\) cds and +31 to +315 of the \(KINDE1\) cds. \(KINDE1\) and \(KINDE2\) are the genes encoding the two \(K.\) \(lactis\) external alternative dehydrogenases (Tarrio et al., 2005; 2006b). The gene of the \(K.\) \(lactis\) actin (KLLA0D05357g) was used as control (positions +1290 to +1728 of the cds). Quantification of the products, separated on agarose gels, stained with ethidium bromide and photographed with a digital system, was performed in tagged image file format with densitometry analysis software (ImageMaster Total Laboratory, version 2.00, GE Healthcare).

**Analysis of expression by qRT-PCR**

Total RNA isolated using the AURUM kit (Bio-Rad) was converted into cDNA and labelled with the KAPA SYBR FAST universal one-step qRT-PCR kit (Kappa Biosystems, Woburn, MA, USA).

PCR primers for \(KINDI1\) were designed with the tool ‘Primer Blast’ at NCBI to generate 80–120 base pairs amplicon. Primer length of 18–24 bp, Tm of 59 or 60°C and G/C content not higher than 50% were selected. The sequence of primers chosen is GAATACTTGGCTAA and G/C content not higher than 50% were selected. The specificity of the primers for the \(KINDI1\) cds was verified.

The ECO Real-Time PCR System was used for the experiments (Illumina, San Diego, CA, USA) and calculations were made by the 2^{-\Delta\Delta Ct} method (Livak and Schmittgen, 2001). Two independent cultures and RNA extractions were assayed for each strain or condition. The mRNA levels of the \(KINDI1\) gene were corrected by the geometric mean of the mRNA levels of the gene \(ACT1\), a control gene also used for RT-PCR experiments as above described, which was previously verified to be constitutive in the assayed conditions. A \(t\)-test was applied to evaluate the differences between \(\Delta Ct\) values (\(\Delta Ct\) values normalized with reference genes) of control and treated samples with a \(P\) value of 0.05.

**Cell respiration rate**

The respiration rate of \(K.\) \(lactis\) PM5-3c and \(Klndi1\) null mutant cells was measured at 25°C using a Clark-type oxygen electrode (Hansatech) following the procedure previously described (García-Leiro et al., 2010a). The cells grown in liquid YPD medium were collected by centrifugation.
(previously \( \text{OD}_{600} \) was measured) and suspended in one-tenth volume of 50 mM potassium phosphate (pH 6.5) buffer with 0.1 M glucose or ethanol. 50–100 \( \mu l \) of the cell suspension was introduced into the chamber filled with 1 ml of the same buffer and the amount of oxygen consumed was recorded. Data are expressed as nmol \( O_2 \) ml\(^{-1} \) min\(^{-1} \) \( \text{OD}_{600}^{-1} \).

**Mitochondrial respiration rate in permeabilized cells**

Five millilitres of \( K. \text{lactis} \) cells of the wild-type PM5-3c and \( \text{Klndi1} \) null mutant strains grown in liquid medium (10 ml of culture in a 50 ml Erlenmeyer flask incubated at 28°C and 250 r.p.m.) up to \( \text{OD}_{600} = 1 \) were collected by centrifugation and suspended in 5 ml of phosphate buffer 25 mM pH = 7.2 with MgCl\(_2\) 5 mM and sorbitol 0.65 M. For permeabilization 0.1 ml of 50–x protease inhibitor Complete Mini, EDTA Free (Roche) and 0.025 ml of digitonin 10 mM were added, tubes were gentle agitated during 1 min, cells collected again by centrifugation and suspended in 0.5 ml of the same buffer.

The respiration rate (oxygen uptake) of permeabilized cells was measured at 28°C using a Clark-type oxygen electrode (Hansatech). In the electrode cuvette, 0.8 ml of buffer was added plus 0.05 ml of adenosine diphosphate (ADP) 50 mM and 0.05 ml of the substrate (1 M pyruvate-malate or 2 M ethanol). After baseline stabilization, 0.1 ml of the permeabilized cell suspension was added and the amount of oxygen consumed was recorded. The basal respiration of cells in the same conditions but without substrate was subtracted. Data are expressed as nmol \( O_2 \) ml\(^{-1} \) min\(^{-1} \). All respiration was found to be sensitive to antimycin A.

**Glucose, lactose and ethanol**

Analyses were done as previously described (González-Siso and Suárez Doval, 1994) with a refractive index detector coupled to an isocratic high-performance liquid chromatography system operating with a Sugar-Pack column (Waters, Millipore) at 90°C, Milli-Q water was the mobile phase at 0.5 ml min\(^{-1} \).

**DNA and nuclear fragmentation**

To evaluate genomic DNA and nuclear integrity, cultures of \( K. \text{lactis} \) PM5-3c and \( \text{Klndi1} \) null mutant in liquid YPD media (2% glucose) were inoculated at \( \text{OD}_{600} = 0.1 \) from fresh overnight pre-cultures every day until 4 days an aliquot was taken. Genomic DNA was extracted and visualized after electrophoresis on agarose gels and ethidium bromide staining. Nuclear staining was performed, after fixing the yeast cells with 2.5% glutaraldehyde on ice for 10 min, with 10 \( \mu l \) propidium iodide and 2.5 \( \mu l \) 4,6-diamidino-2-phenylindole. The stained cells were viewed with a fluorescent microscope and photographed with a digital camera.

**Life span**

Chronological life span of \( K. \text{lactis} \) PM5-3c and \( \text{Δklndi1} \) mutant was determined by measurement of colony-forming ability [colony-forming units (cfu)]. Cultures in liquid YPD and CM media with 2% glucose (two cultures in YPD and two cultures in CM) were inoculated at \( \text{OD}_{600} = 0.1 \) from fresh overnight pre-cultures in the same medium; after 2 days of growth, the cells were washed and transferred to distilled water at \( \text{OD}_{600} = 1 \) and further incubated in the same conditions (at 30°C and 150–250 r.p.m.). The first sample was taken at the moment of transfer and considered 100% viability. Aliquots of the cultures containing approximately 100 cells were plated to YPD medium. Every 2 days, new samples were taken to score viability until less than 10% of the culture was viable. The half-life of each strain was calculated by exponential fit of the data with Excel software.

**Isolation of mitochondria**

\( Kluveromyces \text{lactis} \) mitochondria were isolated as previously described (Tarrío et al., 2005; 2006b). Cells were aerobically grown in CM with 1% glucose as carbon source.

**Oxygen uptake studies with mitochondrial preparations**

Substrate-dependent oxygen consumption rates of freshly isolated mitochondria were determined at 30°C using a Clark-type oxygen electrode (Hansatech) as previously described (Tarrío et al., 2005; 2006b). Respiratory substrates were NADH (0.2 mM) and pyruvate + L-malate (5 mM), which generate intramitochondrial NADH. Measurements were made in the absence or presence of 0.25 mM ADP, and all respiration was found to be sensitive to antimycin A.

**Bioethanol production from cheese whey**

Cheese whey ultrafiltered permeate, threefold concentrated, was obtained from a local cheese factory (Queizúar S.L.). Cultures of wild type and \( \text{Klndi1} \) null mutant strains were performed in a 2 l vessel bioreactor (Biostat MD). Preinocula were grown in YPD at 30°C. Temperature, pH, agitation and aeration were maintained at 30°C, 5.6, 500 r.p.m. and 1–1.3 l min\(^{-1} \) respectively for 40 h when aeration was stopped and agitation reduced to 150 r.p.m. Samples were taken at determined time intervals for analysis of growth (\( \text{OD}_{600} \)), beta-galactosidase activity, lactose and ethanol.

For cultures in sealed tubes, preinocula were grown in 500 ml flasks at 30°C in 200 ml YPD. When cells reached an \( \text{OD}_{600} = 3 \) at 600 nm, they were centrifuged and added as inoculum to 100 ml sealed tubes containing 40 ml of three-times concentrated cheese whey permeate. They were grown at 30°C and 250 r.p.m. Aliquots were taken at different times for analysis of growth (\( \text{OD}_{600} \)), beta-galactosidase activity, lactose and ethanol.
Beta-galactosidase activity

Beta-galactosidase activity was measured using the method of Guarente (1983) as previously described (Becerra et al., 2001). One enzyme unit (EU) was defined as the quantity of enzyme that catalyzes the liberation of 1 nanomol of orthonitrophenol from orthonitrophenyl-β-D-galactopyranoside per min under assay conditions. EU are expressed per ml of culture medium.

Protein determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

Other procedures

Standard procedures for manipulation of nucleic acids were essentially those of Sambrook and colleagues (1989). Escherichia coli DH-10B was used for plasmid amplification. Yeast transformation was performed using a lithium acetate procedure (Gietz and Woods, 1994).

Statistical analysis

Data are expressed as mean ± standard deviation. The statistical significance of differences found between means was evaluated by the unpaired Student’s t-test, two-tailed P values calculated with Excel software.

Acknowledgements

We thank Dr. M. Wésolewski-Louvel for providing the strain PM5-3c and QUEIZUAR S.L. for the cheese whey. Aida Sánchez rendered technical assistance. We also thank Mariana Fernández, Ana García and Fátima Rodríguez for collaboration in preliminary studies with this mutant.

References

Arif, K., Islam, M.A., Marof, A., Shazia, K., Umber, K., Deepa, B., and Owais, M. (2008) Applications of whey in biotechnology. In Advances in Cheese Whey Utilization. Cerdán, M.E., González-Siso, M.I., and Becerra, M. (eds). Kerala, India: Transworld Research Network, pp. 1–34.

Bakker, B.M., Bro, C., Kötter, P., Luttik, M.A.H., Van Dijken, J.P., and Pronk, J.T. (2000) The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in Saccharomyces cerevisiae. J Bacteriol 182: 4730–4737.

Bakker, B.M., Overkamp, K.M., Van Maris, A.J.A., Kötter, P., Luttik, M.A.H., Van Dijken, J.P., and Pronk, J.T. (2001) Stoichiometry and compartmentation of NADH metabolism in Saccharomyces cerevisiae. FEMS Microb Rev 25: 15–37.

Balaban, R.S., Remoto, S., and Finkel, T. (2005) Mitochondria, oxidants and aging. Cell 120: 483–495.

Becerra, M., Díaz Prado, S., González-Siso, M.I., and Cerdán, M.E. (2001) New secretory strategies for Kluyveromyces lactis beta-galactosidase. Protein Eng 14: 379–386.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Cui, Y., Zhao, S., Wu, Z., Dai, P., and Zhou, B. (2012) Mitochondrial release of the NADH dehydrogenase Ndi1 induces apoptosis in yeast. Mol Biol Cell 23: 4373–4382.

De Ris, J.L., Iyer, V.R., and Brown, P.O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278: 680–686.

Della-Bianca, B.E., Basso, T.O., Stambuk, B.U., Basso, L.C., and Gombert, A.K. (2013) What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol 97: 979–991.

Diniz, R.H.S., Rodrigues, M.Q.R.B., Fietto, L.G., Passos, F.M.L., and Silveira, W.B. (2013) Optimizing and validating the production of ethanol from cheese whey permeate by Kluyveromyces marxianus UFV-3. Biocatal Agric Biotechnol 3: 111–117.

Duarte, M., and Videira, A. (2007) Mitochondrial NAD(P)H dehydrogenases in filamentous fungi. In Complex I and Alternative Dehydrogenases. González-Siso, M.I., and Cerdán, M.E. (eds). Kerala, India: Transworld Research Network, pp. 55–68.

García-Leiro, A., Cerdán, M.E., and González-Siso, M.I. (2010a) A functional analysis of Kluyveromyces lactis glutathione reductase. Yeast 27: 431–441.

García-Leiro, A., Cerdán, M.E., and González-Siso, M.I. (2010b) Proteomic analysis of the oxidative stress response in Kluyveromyces lactis and effect of glutathione reductase depletion. J Proteome Res 9: 2358–2376.

Gietz, R.D., and Woods, R.A. (1994) High efficiency transformation in yeast. In Molecular Genetics of Yeast: Practical Approaches. Johnston, J.A. (ed.). Oxford, UK: Oxford University Press, pp. 121–134.

González-Siso, M.I. (1996) The biotechnological utilization of cheese whey: a review. Biorec Technol 57: 1–11.

González-Siso, M.I., and Cerdán, M.E. (2007) Mitochondrial alternative NAD(P)H-dehydrogenases and respirofermentative metabolism in yeasts. In Complex I and Alternative Dehydrogenases. González-Siso, M.I., and Cerdán, M.E. (eds). Kerala, India: Transworld Research Network, pp. 69–84.

González-Siso, M.I., and Suárez Doval, S. (1994) Kluyveromyces lactis immobilization on corn grits for milk whey lactose hydrolysis. Enzyme Microb Technol 16: 303–310.

González-Siso, M.I., Ramil, E., Cerdán, M.E., and Freire Picos, M.A. (1996a) Respirofermentative metabolism in Kluyveromyces lactis: ethanol production and the Crabtree effect. Enzyme Microb Technol 18: 585–591.

González-Siso, M.I., Freire-Picos, M.A., and Cerdán, M.E. (1996b) Reoxidation of the NADPH produced by the pentose phosphate pathway is necessary for the utilization of glucose by Kluyveromyces lactis rag2 mutants. FEBS Lett 387: 7–10.
González-Siso, M.I., Freire-Picos, M.A., Ramil, E., González-Domínguez, M., Rodríguez-Torres, A., and Cerdán, M.E. (2000) Respirofermentative metabolism in Kluyveromyces lactis: insights and perspectives. *Enzyme Microb Technol* **26**: 699–705.

González-Siso, M.I., Pereira, A., Fernández Leiro, R., Cerdán, M.E., and Becerra, M. (2008) Production of recombinant proteins from cheese whey. In *Advances in Cheese Whey Utilization*. Cerdán, M.E., González-Siso, M.I., and Becerra, M. (eds). Kerala, India: Transworld Research Network, pp. 195–209.

González-Siso, M.I., García-Leiro, A., Tarrío, N., and Cerdán, M.E. (2009) Sugar metabolism, redox balance and oxidative stress response in the respiratory yeast *Kluyveromyces lactis*. *Microb Cell Fact* **8**: 46.

Guarante, L. (1983) Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol* **101**: 181–191.

Guimarães, P.M.R., Teixeira, J.A., and Domingues, L. (2010) Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. *Biotechnol Adv* **28**: 375–384.

Hacioglu, E., Demir, A.B., and Koc, A. (2012) Identification of respiratory chain gene mutations that shorten replicative life span in yeast. *Exp Gerontol* **47**: 149–153.

Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics, A Cold Spring Harbor Laboratory Course Manual*. New York, NY, USA: Cold Spring Harbor Laboratory Press.

Li, W., Sun, L., Liang, Q., Wang, J., Mo, W., and Zhou, B. (2006) Yeast AMID homologue Ndp1p displays respiration-restricted apoptotic activity and is involved in chronological aging. *Mol Biol Cell* **17**: 1802–1811.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔCT) method. *Methods* **25**: 402–408.

Luttik, M.A.H., Overkamp, K.M., Kötter, P., de Vries, S., van Dijken, J.P., and Pronk, J.T. (1998) The *Saccharomyces cerevisiae NDE1* and *NDE2* genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. *J Biol Chem* **38**: 24529–24534.

Marella, M., Seo, B.B., Nakamaru-Ogiso, E., Greenamyre, J.T., Matsuno-Yagi, A., and Yagi, T. (2008) Protection by the NDI1 Gene against neurodegeneration in a rotenone model. *PLoS ONE* **3**: e1433.

Marella, M., Seo, B.B., Thomas, B.B., Matsuno-Yagi, A., and Yagi, T. (2010) Successful amelioration of mitochondrial optic neuropathy using the yeast NDI1 gene in a rat animal model. *PLoS ONE* **5**: e11472.

Marcos, C.A., de Vries, S., and Grivell, L.A. (1991) Isolation and inactivation of the nuclear gene encoding the rotenone-insensitive internal NADH:ubiquinone oxidoreductase of mitochondria from *Saccharomyces cerevisiae*. *Eur J Biochem* **195**: 857–862.

Moeller, I.M. (2002) A new dawn for plant mitochondrial NAD(P)H dehydrogenases. *Trends Plant Sci* **7**: 235–237.

Oliveira, G.A., Tahara, E.B., Gombert, A.K., Barros, M.H., and Kowaltowski, A.J. (2008) Increased aerobic metabolism is essential for the beneficial effects of caloric restriction on yeast life span. *J Bioenerg Biomembr* **40**: 381–388.

Rigoulet, M., Aguilaniu, H., Avéret, N., Bunoust, O., Camougrand, N., Grandier-Vazeille, X., *et al.* (2004) Organization and regulation of the cytosolic NADH metabolism in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biochem* **256**: 73–81.

Rodicio, R., and Heinisch, J.J. (2013) Yeast on the milky way: genetics, physiology and biotechnology of *Kluyveromyces lactis*. *Yeast* **30**: 165–177.

Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeasts. *Methods Enzymol* **194**: 281–301.

Salioła, M., D’Amici, S., Spontiello, M., Mancini, P., Tassone, P., and Falcone, C. (2010) The transdehydrogenase genes *KINDE1* and *KINDE2* regulate the expression of the *KIGUT2* in the yeast *Kluyveromyces lactis*. *FEMS Yeast Res* **10**: 518–526.

Sambrook, J., Maniatis, T., and Fritsch, E.F. (1989) *Molecular Cloning: A Laboratory Manual*. New York, NY, USA: Cold Spring Harbor Laboratory Press.

Szczo-drak, J., Szewczuk, D., Rogalski, J., and Fiedurek, J. (1997) Selection of yeast strain and fermentation conditions for high-yield ethanol production from lactose and concentrated whey. *Acta Biotechnol* **17**: 51–61.

Tarrío, N., Diaz-Prado, S., Cerdán, M.E., and González-Siso, M.I. (2005) The nuclear genes encoding the internal (*KINDE1*) and external (*KINDE2*) alternative NAD(P)H:ubiquinone oxidoreductases of mitochondria from *Kluyveromyces lactis*. *BBA – Bioenergetics* **1707**: 199–210.

Tarrío, N., Becerra, M., Cerdán, M.E., and González-Siso, M.I. (2006a) Reoxidation of cytosolic NADPH in *Kluyveromyces lactis*. *FEMS Yeast Res* **6**: 371–380.

Tarrío, N., Cerdán, M.E., and González-Siso, M.I. (2006b) Characterization of the second external alternative dehydrogenase from mitochondria of the respiratory yeast *Kluyveromyces lactis*. *BBA – Bioenergetics* **1757**: 1476–1484.

Tarrío, N., García-Leiro, A., Cerdán, M.E., and González-Siso, M.I. (2008) The role of glutathione reductase in the interplay between oxidative stress response and turnover of cytosolic NADPH in *Kluyveromyces lactis*. *FEMS Yeast Res* **8**: 597–606.

Węsolski-Louvel, M., Prior, C., Borneccque, D., and Fukuhara, H. (1992) Rag mutations involved in glucose metabolism in yeast: isolation and genetic characterization. *Yeast* **8**: 711–719.

Yagi, T., Seo, B.B., Nakamaru-Ogiso, E., Marella, M., Barber-Singh, J., Yamashita, T., and Matsuno-Yagi, A. (2006) Possibility of transkingdom gene therapy for Complex I diseases. *BBA – Bioenergetics* **1757**: 708–714.

Zaragoza, O. (2004) Generation of disruption cassettes in vivo using a PCR product and *Saccharomyces cerevisiae*. *J Microbiol Methods* **52**: 141–145.

Zitomer, R.S., and Hall, B.D. (1976) Yeast cytochrome c messenger RNA. *In vitro* translation and specific immunoprecipitation of the *CYC1* gene product. *J Biol Chem* **251**: 6320–6326.