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Carbon catabolite repression in *Kluyveromyces lactis*: isolation and characterization of the *KlDLD* gene encoding the mitochondrial enzyme D-lactate ferricytochrome *c* oxidoreductase

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Abstract In the "petite-negative" yeast *Kluyveromyces lactis* carbon catabolite repression of some cytoplasmic enzymes has been observed. However, with respect to mitochondrial enzymes, in *K. lactis*, unlike the case in the "petite-positive" yeast *Saccharomyces cerevisiae*, growth on fermentable carbon sources does not cause repression of respiratory enzymes. In this paper data are reported on carbon catabolite repression of mitochondrial enzymes in *K. lactis*, in particular on L- and D-lactate ferricytochrome *c* oxidoreductase (LCR). The L- and D-LCR (E.C. 1123, E.C. 1124) in yeast catalyze the stereospecific oxidation of D and L isomers of lactate to pyruvate. This pathway is linked to the respiratory chain, cytochrome *c* being the electron acceptor of the redox reaction. We demonstrate that the level of mitochondrial D- and L-LCR is controlled by the carbon source, being induced by the substrate lactate and catabolite-repressed by glucose. We cloned the structural gene for D-LCR of *K. lactis* (*KlDLD*), by complementation of growth on D,L-lactate in the *S. cerevisiae* strain WWF18-3D, carrying both a *CYB2* disruption and the *dld* mutation. From the sequence analysis an open reading frame was identified that could encode a polypeptide of 579 amino acids, corresponding to a calculated molecular weight of 63 484 Da. Analysis of mRNA expression indicated that glucose repression and induction by lactate are exerted at the transcriptional level.

Key words *Kluyveromyces lactis* · Catabolite repression Mitochondrial enzyme · Lactate dehydrogenase

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Introduction

In microorganisms, growth on glucose causes repression of the synthesis of several enzymes required for the metabolism of either fermentable or non-fermentable carbon sources. This phenomenon is known as carbon catabolite repression (Magasanik 1961). In the yeast *Saccharomyces cerevisiae*, this regulatory mechanism has been extensively studied and a multitude of genes involved have been identified and characterized (for a review see Gancedo 1992). Cytoplasmic as well as mitochondrial enzymes can be affected. Respiratory enzymes belong to the class of repressed enzymes. In this way, in the "petite-positive" yeast *S. cerevisiae*, fermentative metabolism supports energetic requirements during growth on glucose.

The mechanism of carbon catabolite repression is also functional in non-*Saccharomyces* yeasts. In the "petite-negative" (Bulder 1964) yeast *Kluyveromyces lactis*, carbon catabolite repression of some cytoplasmic enzymes such as NAD glutamate dehydrogenase and β-galactosidase has been observed (Ferrero *et al.* 1978; Breunig 1989; Kuzhandaivelu et al. 1992). It has also been reported that there is a strain-dependent sensitivity to glucose repression. (Ferrero et al. 1978; Breunig 1989; Kuzhandaivelu et al. 1992).

As far as mitochondrial enzymes are concerned, in *K. lactis* growth on fermentable carbon sources does not cause repression of respiratory enzymes (DeDeken 1966). However, some form of regulation exerted by glucose on mitochondrial functions does exist, since induction by glucose of mitochondrial, antimycin A-insensitive respiration has been reported (Ferrero et al. 1981; Lodi et al. 1985).

To obtain more information about the regulatory effect of catabolite repression on mitochondrial enzymes in *K. lactis*, we have undertaken a physiological study of the lactate ferricytochrome *c* oxidoreductase system (LCR). The L- and D-LCR (E.C. 1123, E.C. 1124)

in yeast catalyze the stereospecific oxidation of L and D isomers of lactate to pyruvate. This pathway is linked to the respiratory chain, cytochrome *c* being the electron acceptor of the redox reaction. This enzymatic system has been extensively characterized in *S. cerevisiae*. The L-LCR (cytochrome *b*2) is a flavohemoprotein (Guiard and Lederer 1976; Jacq and Lederer 1974; Lederer et al. 1985); the D-LCR is a Zn flavoprotein (Nygaard 1961; Gregolin and Singer 1962, 1963). Both are nuclear encoded and localized in mitochondria, but the mechanism of import into the organelles has been elucidated only for cytochrome *b*2 (Gasser et al. 1982, Pfanner et al. 1988; Glick et al. 1992; Koll et al. 1992). The synthesis of the two enzymes is subject to the negative control of catabolite repression (Galzy and Slonimski 1957; Somlo 1965). Moreover, both enzymes are absent in anaerobic cells and are induced during respiratory adaptation; both enzymes are induced by either of the two isomers of lactate (Slonimski 1953; Labeyrie and Slonimski 1964; Solmo 1965, 1966, 1967; Lodi and Guiard 1991).

Here we describe the LCR system in the yeast *K. lactis* and demonstrate that the synthesis of mitochondrial D- and L-LCR is controlled by carbon sources added during cell growth. In particular, unlike cytochromes or other respiratory enzymes, D- and L-LCR expression is affected by glucose. Moreover, cloning and molecular study of the structural gene of D-LCR of *K. lactis* is reported.

Materials and methods

Strains

The *S. cerevisiae* strain used in this study, WWF18-3D (**a** *ade2 leu2 his3 ura3 CYB2*::*TRP1 dld*), was previously characterized (Lodi and Ferrero 1993). The *K. lactis* strain PM4–4B (α *uraA ade* pKD1$^+$) has been described elsewhere (Goffrini et al. 1991).

Escherichia coli strains JM83 *ara* Δ(*lac-proAB*) *rpsL* (= *strA*) *ϕ 80 lacZ* Δ*M15* and MC1066 (Casadaban et al. 1983) were used for plasmid amplification and preparation.

Media

M medium contained 5 g/l Difco yeast extract and 10 g/l Difco Bactopeptone supplemented with the carbon sources indicated in results. Mineral minimal medium was supplemented with 20 g/l glucose, and appropriate amino acids, uracil, or adenine to a final concentration of 40 μg/ml.

The E. coli strains were grown in LB or M9 broth (Maniatis et al. 1982). Ampicillin was added to a final concentration of 150 μg/ml for plasmid maintenance.

Isolation of mitochondria

Three milliliters of extraction buffer (0.6 M sorbitol, 10 mM imidazole, 0.5 mM EDTA and 0.1% BSA, pH 6.4) and 3 g of glass beads (0.5 mm diameter) were added to each gram of wet weight cells. The cells were broken by vortexing at 4° C. The suspension was then centrifuged at 5000 g for 5 min, the precipitate discarded and the supernatant centrifuged at 17000 *g* for 30 min. The mitochondrial pellet was washed once and resuspended in the extraction buffer.

Preparation of cell free extract

Cells grown to 1×10^8 cells/ml were harvested by centrifugation, washed once with distilled water and once with extraction buffer [0.03 M TRIS-HCL, pH 8.5, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell pellet was resuspended in 1.25 volumes of extraction buffer, and cells were disrupted by vortexing at 4° C in the presence of an equal volume of glass beads (0.5 mm diameter). The suspension was then centrifuged at 5000 *g* for 5 min at 4° C. The pellet was discarded, and the supernatant used for enzyme activity measurements.

Enzyme assays

L- and D-LCR activities were measured spectrophotometrically at 600 nm at a standard temperature of 30° C in the presence of 50 mM phosphate buffer, pH 7, 10 mM sodium azide, 30 μM phenazine methosulfate, 50 μM 2,6 dichlorophenol-indophenol, 30 μM lactate (lithium salt) and an amount of mitochondria (for D-LCR assay) or cell-free extract (for L-LCR assay) corresponding to approximately 0.1 mg/ml in the reaction mixture. The exogenous substrate was added to the reaction mixture after exhaustion of endogenous substrates.

Measurement of respiratory activity

This assay was carried out according to Ferrero et al. (1981).

Transformation

Transformation of *S. cerevisiae* cells was performed according to Ito et al. (1983). Transformants were selected on mineral minimal medium containing 2% glucose and the required amino acids or bases. *K. lactis* transformation was carried out according to Bianchi et al. (1987). Transformation of *E. coli* was performed according to Mandel and Higa (1970).

Plasmids

The genomic library of fragments of partially *Sau*3A digested *K. lactis* DNA in pSK1 was provided by M. Wésolowski-Louvel. The plasmid pFL44 was provided by F. Lacroute; plasmid pUC19 was purchased from Pharmacia.

Preparation, analysis of DNA and electrophoresis

Isolation and purification of plasmids from *E. coli* were carried out by standard methods (Maniatis et al. 1982). Established methods were used for preparation of yeast DNA (Nasmyth and Reed 1980). Agarose gel electrophoresis and Southern analysis were as described elsewhere (Maniatis et al. 1982).

Enzymes and chemicals

Restriction endonucleases and T4 DNA ligase were purchased from Pharmacia. Digestion and ligation were performed in accordance with the supplier's instructions. ^{32}P-labeled substrates for DNA probes were purchased from NEN-DU PONT.

Nucleotide sequence analysis

Nucleotide sequence determination of the *KlDLD* gene was carried out by using the dideoxy chain termination method (Sanger et al. 1977) with Sequenase Version 2 (USB). Overlapping deletion subclones were produced by digestion with exonuclease III and S1 nuclease (Nested Deletion kit, Pharmacia) starting from opposite ends of the DNA insert. The sequence of both strands was determined by using the double-stranded plasmid DNA as a template and the 17-nucleotide universal and reverse sequencing primers (Pharmacia).

Northern analysis

Total RNA isolation and formamide agarose gel electrophoresis were carried out according to Sherman et al. (1986). Northern blotting to Hybond N filters (Amersham) and hybridization to ^{32}P-labeled probes were performed using standard techniques (Maniatis et al. 1982).

Results

Effects of growth on different carbon sources on levels of the mitochondrial enzymes D- and L-LCR in *K. lactis*

The effects of fermentative and oxidative carbon sources on the levels of D-LCR and L-LCR were analyzed in *K. lactis*. L-LCR was tested in cell-free extracts and D-LCR was tested in isolated mitochondria. Both L- and D-LCR are in fact mitochondrial enzymes, but L-LCR is a soluble protein located in the intermembrane space, whereas D-LCR is linked to the membrane (Somlo 1965; Lodi and Ferrero 1993). Table 1 reports the enzyme activities measured in cultures grown, respectively, in the presence of D-, L-lactate, ethanol and glucose, alone or in the presence of D-, L-lactate, together with the oxygen consumption data obtained under the same growth conditions. Each carbon source was supplemented to a final concentration of 2%.

Table 1 Effects of growth on different carbon sources on levels of L- and D-lactate ferricytochrome *c* oxidoreductases (L-LCR, D-LCR) and respiratory activity in the strain PM4-4B. L- and D-LCR activities and oxygen consumption (Q_{O_2}) were measured as described in Materials and methods. D-LCR was measured after isolation of mitochondria; L-LCR was measured in cell-free extracts. Enzyme units are expressed as nanomoles of substrate per minute per milligram of protein. Q_{O_2} is expressed as microliters of oxygen consumed per hour per milligram of dry mass. All the values in the Table represent the average of three independent experiments. In no case was the variation higher than 15%

	Growth conditions			
	2% ethanol	2% lactate	2% glucose	2% glucose + 2% lactate
L-LCR	150	695	118	122
D-LCR	394	1505	206	258
Q_{O_2}	78	80	83	82

After growth on ethanol, considered as a non-repressing carbon source, the level of L-LCR was only barely higher than on glucose; the level of D-LCR was approximately twice the level observed on glucose. Lactate, the substrate of L- and D-LCR, caused induction of both enzymes, which was prevented by glucose. The level of respiratory activity was the same, independent of the carbon sources added in the medium. Therefore, both D- and L-LCR, unlike the respiratory enzymes, are subject to glucose catabolite repression, which is epistatic to lactate induction.

Cloning of the *KlDLD* gene

In an attempt to clone the genes coding for D- and L-LCR in *K. lactis* we took advantage of a previously constructed strain of *S. cerevisiae*, WWF18-3D, which carries a non-reverting mutation (obtained by gene disruption) in the *CYB2* gene, coding for L-LCR, and a mutation in *DLD* gene, coding for D-LCR (Lodi and Ferrero 1993). These mutations eliminate both LCR enzyme activities and the strain cannot grow on medium supplemented with D, L-lactate (Lct$^-$ phenotype).

The WWF18-3D strain was transformed with a *K. lactis* genomic library, kindly provided by Dr. M. Wésolowski-Louvel (Wésolowski-Louvel et al. 1988) and composed of DNA fragments generated by partial *Sau*3A digestion and inserted into the *Bam*HI site of the vector pSK1. Of about 10000 Ura$^+$ transformants obtained in three different experiments, and replica-plated onto M medium supplemented with D-, L-lactate, two possessed a Lct$^+$ phenotype. When these clones were grown under non-selective conditions, the Ura$^+$ and the Lct$^+$ phenotypes were always lost together in subclones. When tested for LCR activities, these clones showed a high level of D-LCR activity only, suggesting that we had isolated the *DLD* gene of *K. lactis* (*KlDLD*).

Plasmids extracted from the two Lct$^+$ clones and amplified in *E. coli* showed different restriction patterns with some common bands. It therefore seems likely that the same gene had been independently cloned twice with different lengths of adjacent DNA. The shorter plasmid, designated pLD3 and containing a 7.2 kb insert, was analyzed further.

In order to localize the *KlDLD* gene in the cloned DNA, different fragments of the plasmid pLD3 were cloned into the pFL44 vector (Bonneaud et al. 1991). The resulting plasmids were tested for their ability to complement the *dld* mutation in the strain WWF18-3D both for growth on lactate and for D-LCR enzyme activity. In this way we delimited the complementing region of pLD3 to a 3.85 kb fragment contained in the

pNSD6 plasmid. The subcloning experiments and restriction map are illustrated in Fig. 1.

Sequence of the *KlDLD* gene

Nucleotide sequence determination of *KlDLD* gene was carried out by the method of Sanger et al. (1977), as described in Materials and methods. An open reading frame (ORF) of 1737 nucleotides was identified by translating the sequenced DNA in the six possible reading frames. The deduced protein sequence contained 579 amino acids and corresponded to a protein of 63484 daltons. The nucleotide and the predicted amino acid sequences are shown in Fig. 2.

In the upstream region two AT-rich stretches were present at positions −160 (TATATATATATA) and −80 (TATATAATAA). The comparison between the *KlDLD* nucleotide sequence and that of the previously isolated *DLD* gene of *S. cerevisiae* revealed that the putative ATG initiation codon (at position + 1 in the sequence) is followed by a stretch of about 260 nucleotides before a region is reached that shows 80% sequence identity with the *S. cerevisiae* homologue (data not shown). Similarly, comparison of the protein sequences deduced from the *KlDLD* and *S. cerevisiae DLD* genes revealed that from positions 1 to 90 the two amino acid sequences are very different; from amino acid 90 to the end of polypeptide the similarity reaches 82%, with an identity score of 64% (Fig. 3).

The codon bias index (CBI) of the *KlDLD* gene, calculated according to Bennetzen and Hall (1982), is 0.62, indicating that the protein is relatively abundant. In this respect we observe a difference between *K. lactis* and *S. cerevisiae*, since the CBI of the *S. cerevisiae DLD* gene is 0.27 (Lodi and Ferrero 1993). Recent studies concerning codon usage in *K. lactis* have indicated that the pattern of synonymous codon usage in the two species appears to be very similar: highly expressed genes have a high codon usage bias, while genes with a low expression level have a low bias (Lloyd and Sharp 1993). It has been observed that corresponding genes in the two yeasts have similar CBIs. An exception to this rule is represented by genes encoding mitochondrial proteins, which display higher CBI values in *K. lactis*, indicating a higher level of gene expression (Luani et al. 1994).

Gene disruption

To obtain a *K. lactis* mutant devoid of D-LCR enzyme, we first constructed a deletion derivative of the *KlDLD* gene. The 2.7 kb region between the *Kpn*I and *Hin*dIII sites was cloned in pUC19. The 1 kb *Bam*HI fragment was excised and replaced by the *Bgl*II cassette bearing the selectable marker *URA3* of *S. cerevisiae* (Bonneaud et al. 1991), which is able to complement the *uraA* mutation of *K. lactis* (De Louvencourt et al. 1983) (Fig. 1). Inactivation of the chromosomal *KlDLD* gene was achieved using the one-step gene replacement method (Rothstein 1983). The *Kpn*I-*Pst*I linear fragment from plasmid pUC19-*DLD*::*URA3* was used to transform strain PM4-4B *uraA*. Several transformants were selected and four were tested for D-LCR activity. One of these was devoid of the enzyme (strain TD4).

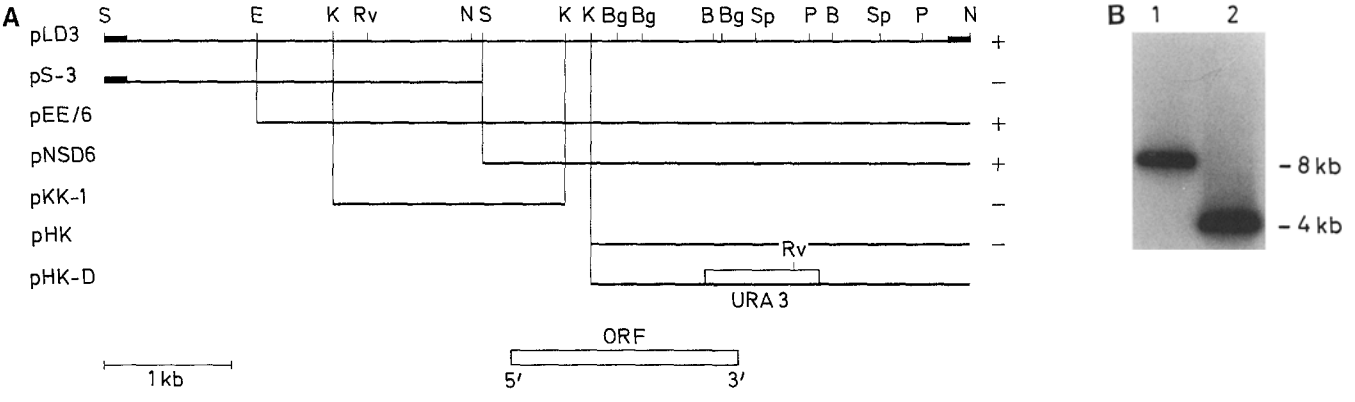

Fig. 1 **A** Restriction enzyme map of plasmid pLD3 containing the *KlDLD* gene and complementation analysis of the derived subclones. The bottom plasmid (pHK-D) represents the selection cassette used for the disruption strategy. Symbols for restriction sites: *B BamHI*; *Bg BglII*; *E EcoRI*; *K KpnI*; *N NheI*; *P PstI*; *RV EcoRV*; *S SalI*; *Sp SpeI*. +, complementation for both growth on lactate and restoration of D-LCR enzyme activity. −, no complementation. The *boxes* indicate pSK1 vector sequences and the *lines* indicate yeast sequences. **B** Confirmation of gene disruption by Southern analysis. Genomic DNA from a wild-type strain (PM4-4B) and a null mutant (TD4 *KlDLD*::*URA3*) was digested with *Eco*RV, subjected to electrophoresis in a 0.8% agarose gel, transferred to a Hybond N membrane and hybridized with ^{32}P-labeled *KlDLD* probe. The *KlDLD* probe hybridized with an 8 kb *Eco*RV fragment in the wild-type genomic DNA (lane 1) and with two fragments of about 4 kb in the DNA of disrupted clones (lane 2) because one *Eco*RV site is present within the *URA3* gene. The lengths of these fragments were deduced from the migration behaviour of co-electrophoresed λ *Hin*dIII fragments. Since the two fragments generated by the deletion of a 1 kb region of the *KlDLD* gene and the insertion of the *URA3* gene (1.2 kb) are of similar length only one band appears in lane 2. Lane 1, wild-type pattern; lane 2, disrupted pattern

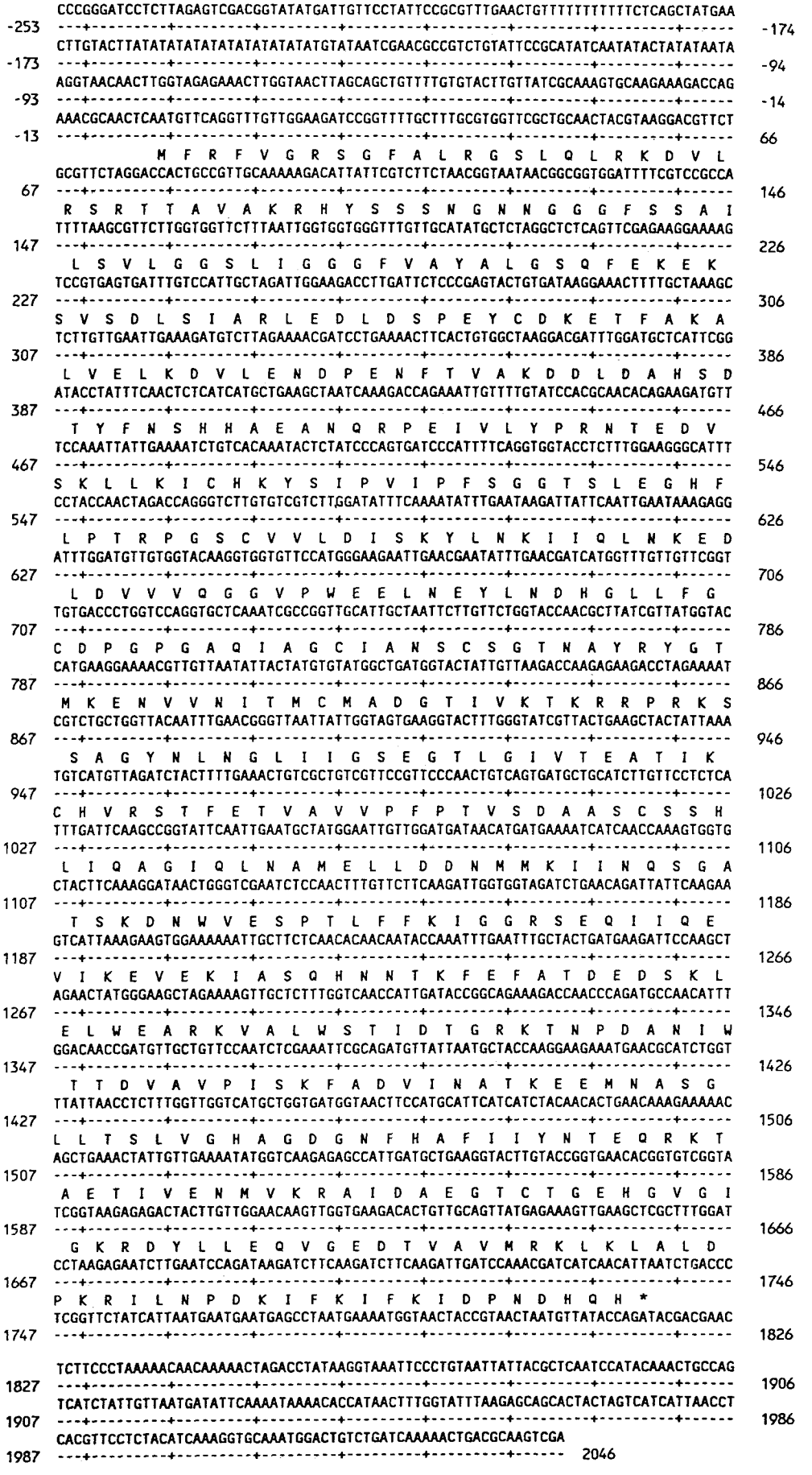

Fig. 2 Nucleotide sequence and predicted amino acid sequence of the *KlDLD* gene. An *asterisk* indicates the termination codon. The genomic sequence has been assigned the accession number X71628 (EMBL/GenBank databases)

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 10 FALRGSLQLRKDVLRSRTTAVAKRHYS.SSNGNNGGGFSSAIL..SVLGG 56
    :  |.: .| |.: ..|.  | :. |. .|.|..:.: ||. |  ||:::
  2 LWKRTCTRLIKPIAQPRGRLVRRSCYRYASTGTGSTDSSSQWLKYSVIAS 51

 57 SLIGGGFV.AYALGSQFEKEKSVSDL.........SIARLEDLDSPEY. 94
    |.  |:: | .| |. .||. :..|         |. :|..||||:|
 52 SATLFGYLFAKNLYSRETKEDLIEKLEMVKKIDPVNSTLKLSSLDSPDYL 101

 95 CDKETFAKALVELKDVLENDPENFTVAKDDLDAHSDTYFNSHHAEANQRP 144
    |. .:.|.: :||:||:|.|||:. ||.||||||||||.||:.::|||
102 HDPVKIDKVVEDLKQVLGNKPENYSDAKSDLDAHSDTYFNTHHPSPEQRP 151

145 EIVLYPRNTEDVSKLLKICHKYSIPVIPFSGGTSLEGHFLPTRPGSCVVL 194
    |:|:|:.||:|||:|||||. .:||:|||||||||||||| |..:.:
152 RIILFPHTTEEVSKILKICHDNNMPVVPFSGGTSLEGHFLPTRIGDTITV 201

195 DISKYLNKIIQLNKEDLDVVVQGGVPWEELNEYLNDHGLLFGCDPGPGAQ 244
    |:||::|.::.::| |||:.||:|:|||:||:||.||||:||||||||||
202 DLSKFMNNVVKFDKLDLDITVQAGLPWEDLNDYLSDHGLMFGCDPGPGAQ 251

245 IAGCIANSCSGTNAYRYGTMKENVVNITMCMADGTIVKTKRRPRKSSAGY 294
    |:|||||||||||||||||||||::|:|:.::|||||||:|||||||||
252 IGGCIANSCSGTNAYRYGTMKENIINMTIVLPDGTIVKTKKRPRKSSAGY 301

295 NLNGLIIGSEGTLGIVTEATIKCHVRSTFETVAVVPFPTVSDAASCSSHL 344
    |||||::|||||||||||||:||||:.. ||||||.|.|:.|||.|.|:|
302 NLNGLFVGSEGTLGIVTEATVKCHVKPKAETVAVVSFDTIKDAAACASNL 351

345 IQAGIQLNAMELLDDNMMKIINQSGATSKDNWVESPTLFFKIGGRSEQII 394
    .|.||:||||||||:||||:||.|:.|.: :|||.||:||||||||..|:
352 TQSGIHLNAMELLDENMMKLINASESTDRCDWVEKPTMFFKIGGRSPNIV 401

395 QEVIKEVEKIASQHNNTKFEFATDEDSKLELWEARKV.ALWSTIDTGRKT 443
    ..::.||. :|  :: ..|:||.|:|.||||||||  |||.:|.::..
402 NALVDEVKAVAQLNHCNSFQFAKDDDEKLELWEARKVRRLWSVLDADKSK 451

444 NPDANIWTTDVAVPISKFADVINATKEEMNASGLLTSLVGHAGDGNFHAF 493
    :..|.|||||||:|.|..||:.||.:|.|| |:..:||||||||||||
452 DKSAKIWTTDVAVPVSQFDKVIHETKKDMQASKLINAIVGHAGDGNFHAF 501

494 IIYNT.EQRKTAETIVENMVKRAIDAEGTCTGEHGVGIGKRDYLLEQVGE 542
    |:|.| |::.|.. :|:.|||||::||||||||||||||:||||::||
502 IVYRTPEEHETCSQLVDRMVKRALNAEGTCTGEHGVGIGKREYLLEELGE 551

543 DTVAVMRKLKLALDPKRILNPDKIF 567
    ..|.:|||:|||:|||||:||:. :
552 APVDLMRKIKLAIDPKRIMNPGQNL 576
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Fig. 3 Sequence alignment between the *KlDLD* gene product and the *DLD* gene product of *Saccharomyces cerevisiae*. *Vertical lines* represent identical amino acid matches; *dots* indicate conserved residues. Gaps introduced for optimal alignment are indicated with *dashes*. Optimal alignment was found using the local homology algorithm of Smith and Waterman by program Bestfit (GCG Wisconsin). The upper sequence corresponds to the *Kluyveromyces lactis* polypeptide

Southern analysis confirmed that gene replacement had taken place.

The utilization of lactate by isogenic strains carrying wild-type or mutant alleles at the *KlDLD* locus was analyzed. Cells grown in M medium supplemented with 0.6% glucose until the carbon source was exhausted, were inoculated at a concentration of 5×10^4 cells/ml into M medium supplemented with either 1% D-lactate or 1% D, L-lactate. In the parental strain, no difference in growth was observed on D-, L-lactate and D-lactate. In the mutant strain, growth on D, L-lactate was observed as for the parental strain, but was not observed on D-lactate alone (data not shown). It thus appears that the enzyme D-LCR is required for the stereospecific utilization of D-lactate and that there is no enzyme activity in *K. lactis* able to convert D-lactate into L-lactate. Moreover, the growth of the mutant strain on D, L- lactate indicates that disruption of the *KlDLD* gene did not affect L-LCR activity.

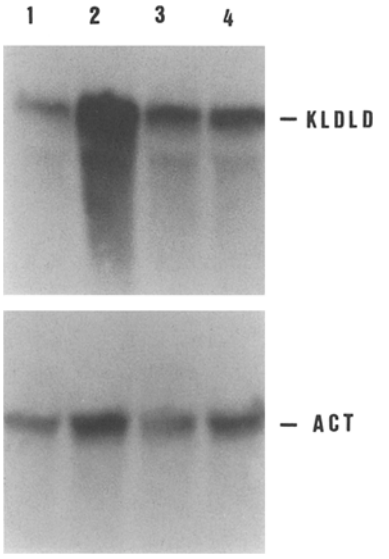

Fig. 4 Northern blot analysis of the *KlDLD* transcripts from strain PM4–4B. Total cellular mRNA was prepared from cells grown on: (1) 2% ethanol; (2) 2% lactate; (3) 2% glucose; (4) 2% glucose plus 2% lactate. Hybridization was carried out using as a probe the *Kpn*I-*Pst*I fragment extracted from pNSD6 and a probe corresponding to the *ACT1* gene of *S. cerevisiae*. The *ACT* mRNA served as an internal standard for the amount of RNA loaded. Each lane contained 20 µg of total mRNA

Transcriptional analysis

To identify the RNA encoded by the *KlDLD* gene and to discover at which level glucose repression and lactate induction are exerted, a Northern analysis of *KlDLD* expression was carried out. Total RNAs were extracted from PM4-4B grown on media supplemented with ethanol, glucose, D, L-lactate with or without glucose. After agarose gel electrophoresis and blotting, the filter was hybridized with the ^{32}P-labeled *Sal*I-*Pst*I fragment corresponding to the *KlDLD* gene.

The Northern analysis (Fig. 4) showed that: (i) the *KlDLD* gene is transcribed as a single hybridizing species of about 1.8 kb, as expected from the sequence analysis; (ii) there is no significant difference between *KlDLD* transcript levels in ethanol and glucose; (iii) the level of *KlDLD* transcript is enhanced when cells are grown on lactate ; (iv) glucose inhibits induction by lactate; in fact the amounts of *KlDLD* mRNA in cells grown on glucose or on lactate plus glucose are the same.

Since, under the different growth conditions analyzed, the rate of transcript production paralleled the level of enzyme activity, we can conclude that the regulation previously observed is exerted mainly at the transcriptional level.

Discussion

The data reported here demonstrate that growth on glucose causes repression of the synthesis of the mitochondrial enzymes L- and D-LCR in the petite-negative yeast *K. lactis*. In contrast, growth on glucose does not affect respiration, which occurs at the same level independently of the carbon source in the growth medium. The existence of carbon catabolite repression of cytoplasmic enzymes in *K. lactis* has previously been reported; here we demonstrate carbon catabolite repression of mitochondrial enzymes.

If we compare the effects of growth on glucose on mitochondrial respiration and on various mitochondrial enzymes in *K. lactis* and in the petite-positive yeast *S. cerevisiae*, it appears that the two species behave differently as far as respiration is concerned (glucose-repressible in *S. cerevisiae*, not glucose-repressible in *K. lactis*), but behave similarly with respect to the enzymes D- and L-LCR, which are repressed in both. However, the effect of carbon catabolite repression of L- and D-LCR in *K. lactis* is different from that in *S. cerevisiae*. In *K. lactis*, glucose repression was mainly observed in the presence of lactate, i.e., under inducing conditions, indicating that glucose inhibits induction by lactate only. In *S. cerevisiae*, glucose repression is exerted both on uninduced and on induced levels, indicating that glucose repression prevents enzyme synthesis and not just its induction (Somlo 1967; Ferrero, unpublished results).

To gain more insight into the carbon catabolite repression of LCRs in *K. lactis*, we undertook the cloning of the genes coding for L- and D-LCR by functional complementation in an *S. cerevisiae* mutant devoid of both LCRs and unable to use lactate as a carbon source. In this way we isolated the structural gene for D-LCR of *K. lactis* (*KlDLD*).

We used the corresponding probe to study the effect of different carbon sources on *KlDLD* gene expression. The highest level of transcripts was observed on lactate. The level of transcripts on ethanol, glucose and glucose plus lactate was quite similar. These results demonstrate that both aspects of regulation of *KlDLD* gene expression, i.e., induction by lactate and repression by glucose operate at the transcriptional level. However it cannot be excluded that post-transcriptional mechanisms might also act that affect metabolic steps that intervene in enzyme import into mitochondria and/or maturation processes.

The *KlDLD* gene was characterized and completely sequenced. The amino acid sequence deduced from the nucleotide sequence of the *KlDLD* gene was compared with that of *S. cerevisiae*, previously characterized. Most of the protein revealed high conservation, except the N-terminal region of approximately 90 amino acids. We propose that the less conserved N-terminal part of the *KlDLD* gene product corresponds to the presequence, necessary to target the protein to mitochondria, which may be cleaved during import into the organelles. The observation that this putative presequence is rich in basic, hydroxylated and hydrophobic residues, typical of the mitochondrial presequences studied to date in *S. cerevisiae* (von Heijne 1986; Roise et al. 1986), favors this hypothesis.

The complementation of an *S. cerevisiae* mutant by the *K. lactis* gene suggests that D-LCR can be imported into heterologous mitochondria, indicating that the Kl-D-LCR putative presequence is able to target the protein into *S. cerevisiae* mitochondria and that the *S. cerevisiae* import machinery is able to process the Kl-D-LCR apoprotein. The ability to express the *KlDLD* gene in *S. cerevisiae* will allow a study of whether and how the *KlDLD* gene of *K. lactis* is regulated in *S. cerevisiae*, contributing to an understanding of the mechanisms of glucose repression in the two species and the differences between them.

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