External alternative NADH:ubiquinone oxidoreductase redirected to the internal face of the mitochondrial inner membrane rescues complex I deficiency in *Yarrowia lipolytica*

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SUMMARY

Alternative NADH:ubiquinone oxidoreductases are single subunit enzymes capable of transferring electrons from NADH to ubiquinone without contributing to the proton gradient across the respiratory membrane. The obligately aerobic yeast *Yarrowia lipolytica* has only one such enzyme, encoded by the *NDH2* gene and located on the external face of the mitochondrial inner membrane. In sharp contrast to *ndh2* deletions, deficiencies in nuclear genes for central subunits of proton pumping NADH:ubiquinone oxidoreductases (complex I) are lethal. We have redirected NDH2 to the internal face of the mitochondrial inner membrane by N-terminally attaching the mitochondrial targeting sequence of NUAM, the largest subunit of complex I. Lethality of complex I mutations was rescued by the internal, but not the external version of alternative NADH:ubiquinone oxidoreductase. Internal NDH2 also permitted growth in the presence of complex I inhibitors such as 2-decyl-4-quinazolinyl amine (DQA). Functional expression of NDH2 on both sides of the mitochondrial inner membrane indicates that alternative NADH:ubiquinone oxidoreductase requires no additional components for catalytic activity. Our findings also demonstrate that shuttle mechanisms for the transfer of redox equivalents from the matrix to the cytosolic side of the mitochondrial inner membrane are insufficient in *Y. lipolytica*.

Key words: Alternative NADH:ubiquinone oxidoreductase, Alternative NADH dehydrogenase, Mitochondrial import, Yeast, *Yarrowia lipolytica*, Mitochondria

INTRODUCTION

In many plants, fungi and prokaryotes electron transfer from NADH to the ubiquinone pool of the respiratory chain can be carried out by two fundamentally different enzymes. Complex I, a large multi subunit enzyme, links this reaction to the translocation of four protons across the membrane (Brandt, 1999). In mammalian mitochondria, where it is the only NADH dehydrogenase, complex I is composed of 43 subunits with a total molecular mass of nearly 1000 kDa (Walker, 1992; Okun et al., 2000; Sazanov et al., 2000). In fungi such as *Neurospora crassa* (Videira, 1998) or *Yarrowia lipolytica* (Djafarzadeh et al., 2000), complex I contains at least 35 subunits. Complex I oxidizes NADH produced mainly by the pyruvate dehydrogenase complex, in the citric acid cycle and in β-oxidation. Therefore, its NADH binding site faces the matrix side of the mitochondrial inner membrane.

By contrast, alternative NADH:ubiquinone oxidoreductases are single subunit enzymes that contain one molecule of non-covalently bound FAD as redox cofactor and do not contribute to the proton gradient across the respiratory membrane. In eukaryotes they may reside either on the external or the internal face of the mitochondrial inner membrane. The physiological significance of this fundamental functional difference within a family of closely related enzymes is unknown. Various plants and fungi are equipped with different sets of alternative NADH:ubiquinone oxidoreductases (Kerscher, 2000). Baker's yeast, *Saccharomyces cerevisiae*, has three alternative NADH:ubiquinone oxidoreductases. Two of these, called SCNDE1 and SCNDE2, are external enzymes (Luttik et al., 1998; Small and McAlister-Henn, 1998), whereas SCNDI1is an internal enzyme (Marres et al., 1991; de Vries et al., 1992). *S. cerevisiae* presents a special case among ascomycetous fungi, since ethanolic fermentation, rather than respiration, is the preferred mode of glucose utilization (Lagunas, 1986). In this organism, complex I is lacking (Büschges et al., 1994) and SCNDI1 is the only NADH:ubiquinone oxidoreductase present within the mitochondrial matrix. By contrast, the obligately aerobic yeast *Y. lipolytica*, which does possess complex I, has only one single alternative enzyme, which is encoded by the *NDH2* gene and resides on the external face of the mitochondrial inner membrane (Kerscher et al., 1999).

These two organisms may illustrate key questions regarding the metabolic function of external and internal alternative NADH:ubiquinone oxidoreductases in ascomycetous fungi: Are there separate metabolic pools for NADH in the cytoplasm and in the mitochondrial matrix or do redox shuttles exist? Do these operate in both directions? Is there competition between

complex I and alternative enzyme(s) for the substrates NADH and ubiquinone? What are the consequences of NADH oxidation via complex I versus via the alternative enzyme? To address such questions, we constructed strains of *Y. lipolytica* that possess either complex I only or complex I and an internal version of alternative NADH:ubiquinone oxidoreductase. We found that the construction of strains that lacked complex I was feasible only when an internal alternative enzyme was present.

MATERIALS AND METHODS

Deletion of the NUAM gene by homologous recombination

The 6.7 kb insert of genomic clone pB7, containing the complete *NUAM* gene encoding the 75 kDa subunit of complex I (Djafarzadeh et al., 2000), was subcloned into a pBluescriptSK$^-$ derivative from which part of the polylinker had been removed by digestion with *Kpn*I and *Cla*I, blunt ending via Klenow DNA polymerase and religation. A part of the *NUAM* open reading frame, corresponding to codon positions 43-596 was removed by digestion with *Kpn*I and *Cla*I and replaced with a 1.6 kb PCR product containing the complete *URA3* gene in opposite orientation to the original *NUAM* open reading frame. A 5.2 kb *Sal*I fragment was used for transformation of diploid GB1 *Y. lipolytica* cells. Integration of the construct by homologous recombination with the wild-type *NUAM* locus in four out of 24 uracil prototrophs was proven by PCR and Southern blot analysis.

Expression of internal YLNDH2

Plasmid pUB4 was constructed from the *Y. lipolytica*/*E. coli* shuttle vector pINA443 (Barth and Gaillardin, 1996) by removing the *URA3* marker by digestion with *Bam*HI and *Sal*I and replacing it with the *Klebsiella pneumoniae* hygromycin B phosphotransferase (*HygB^R*) gene (Gritz and Davies, 1983) under the control of a strong hybrid promoter, consisting of four direct repeats of UAS1B from *Y. lipolytica XPR2* in front of the minimal promoter of *Y. lipolytica LEU2* (Madzak et al., 1999; Madzak et al., 2000). The new marker gene was generated by PCR using primers *Bam*HI/Promc2 (5′-GTGGATCCAGGCCGTTGAGCACC-3′) and Prom/nc (5′-TGTGGATGTGTGTGGTTGTATG-3′) to amplify the hybrid promoter out of plasmid pINA1051 and primers Hyg/c (5′-ATGAAAAAGCCTGAACTCACC-3′) and *Sal*I/Hyg/nc (5′-GTCGTCGACTATTCCTTTGCCCTC-3′) to amplify the *HygB^R* ORF out of plasmid pDHHyg, followed by blunt end ligation and reamplification using primers *Bam*HI/Promc2 and *Sal*I/Hyg/nc. Cloning via *Bam*HI and *Sal*I was followed by the removal of *Aat*II and *Eco*RI sites within the *HygB^R* ORF by site directed mutagenesis according to the QuikChange protocol (Stratagene).

Plasmid pUB22, containing the *NDH2i* fusion gene encoding the internal version of the *Y. lipolytica* alternative NADH:ubiquinone oxidoreductase under the control of the *NUAM* promoter, was generated in the following way: inverse PCR using primers 75kSK1 (5′-GAGACGTCGGGCGGAGAC-3′) and 75kSK2 (5′-GGATAGTTCGAGGATAGTGGAG-3′) on genomic clone pB7 yielded a product that was gapped for the *NUAM* open reading frame except for the presequence (amino acids 1-34). This was blunt-end ligated with another PCR product, encompassing a truncated version of the *NDH2* open reading frame, starting at amino acid position 100, which was changed from Asn to Asp, generated using primers YLNDH2SK1 (5′-GACCCCTCCGACCAGTTGC-3′) and YLNDH2SK2 (5′-AGAGATATCACGGCCGAAGAC-3′) on genomic clone pE8 (Kerscher et al., 1999). In the resulting clone, called pUB5, a *Cla*I site was generated upstream of the *NDH2i* start codon at position –32 to –27 by site-directed mutagenesis, yielding clone pUB5*Cla*I. A 4.3 kb *Sal*I fragment from pUB5*Cla*I, containing the *NDH2i* fusion gene flanked by 1.5 kb and 1.3 kb of upstream and downstream sequence from the *NUAM* locus, respectively, was subcloned into pUB4.

Expression of other alternative NADH:ubiquinone oxidoreductases

To create the *YLNUAM-ECNDH* fusion, genomic clone pB7 was gapped for the *NUAM* open reading frame except for the presequence (amino acids 1-34) by inverse PCR as described above. The product was blunt-end ligated with another PCR product entailing the complete *ECNDH* (Young et al., 1981) open reading frame in which the TTG initiation codon had been replaced by alanine and aspartate codons, generated on *E. coli* genomic DNA using primers ECNDH1 (5′-GCCGAGACTACGCCATTGAAAAAG-3′) and ECNDH2 (5′-ATGCAACTTCAAACGCGGAC-3′). Subcloning of a 4.3 kb *Sal*I fragment into pUB4 yielded plasmid pUB10. A clone with two 4.3 kb *Sal*I inserts in tandem orientation was termed pUB11.

To express the *SCNDI1* gene in *Y. lipolytica*, genomic clone pE8 was gapped for the entire *YLNDH2* open reading frame and 355 bp of upstream sequence by inverse PCR using primers NDH2inv1 (5′-CTCATACGGGCGGTATTAC-3′) and NDH2inv2 (5′-AGAGTTGCAGCTTCTCCATC-3′). The product was blunt-end ligated with another PCR product entailing the complete *SCNDI1* open reading frame under the control of the same strong hybrid promoter that was also used to express the hygromycin B phosphotransferase (*HygB^R*) marker gene of plasmid pUB4. The insert was generated by PCR using primers *Bam*HI/Promc2 and Prom/nc to amplify the hybrid promoter out of plasmid pINA1051, and primers SCNDI1SK1 (5′-ATGCTATCGAAGAATTTGTATAG-3′) and (5′-GTGTTAGGTTTTGTTTAGAGG-3′) to amplify the SCNDI1 open reading frame out of plasmid pMV5 (Marres et al., 1991), followed by blunt-end ligation and reamplification using primers *Bam*HI/Promc2 and SCNDI1SK2.

Mutagenesis of the SCNDI1 processing site, in order to convert the –1 arginine into a –3 arginine by the insertion of two alanine codons behind this arginine codon was carried out by inverse PCR using 5′-phophorylated primers SCNDImut1 (5′-AGCTCTGGTGGAAGCGAATCTGAC-3′) and SCNDImut2 (5′-GCTTCCACAGGGGTGGAAAACTC-3′), followed by religation. Subcloning of a 4.5 kb *SalI* fragment into pUB4 yielded plasmids pUB27 (–3 arginine) and pUB28 (–1 arginine).

Y. lipolytica techniques

Y. lipolytica genetic techniques such as transformation and sporulation were carried out as described (Barth and Gaillardin, 1996). For the determination of growth rates, complete media containing 5 g/l yeast extract, 10 g/l bacto peptone and 10 g/l of glucose or 4 g/l of sodium acetate, adjusted to pH 5.0, were used. 50 μg/ml hygromycin B (Invitrogen) was added after sterilization. 100 ml portions of media were inoculated in 1 l Erlenmeyer flasks with baffled indentations at a density of 1×10^5 cells/ml with cells pre-grown in the same medium and shaken at 220 rpm. OD_{600} was monitored at 4 hour intervals using a Hitachi U1100 spectrophotometer.

RESULTS

Complex I is essential in *Y. lipolytica*

As part of a general approach to establish *Y. lipolytica* as a model system to study the structure and function of complex I by yeast genetics, we tried to construct deletion strains for several of its central subunits. As an example, the strategy for deletion of the *Y. lipolytica NUAM* gene by homologous recombination with a *URA3* marked deletion allele is depicted in Fig. 1. The *NUAM* gene encodes the 75 kDa subunit of respiratory chain complex I, which contains the ligands for iron-sulfur clusters N1b, N5 and N4 (Ohnishi, 1998; Finel, 1998). Although we were able to generate deletions in one of the alleles of *NUAM* and in several other complex I genes (Kashani-Poor et al., 2001; Ahlers et al., 2000a; Ahlers et al.,

2000b) in the diploid strain GB1, we failed to isolate haploid spores carrying these deletion alleles, either by random spore analysis or by ascus dissection. As we also failed to introduce the deletion alleles directly into haploid yeast strains, it seemed that complex I was essential for vegetative growth. However, we could not exclude at this point that complex I might be essential only for sporulation. To test this possibility, the wild-type *NUKM* gene was subcloned into the single copy replicative plasmid pINA443, which carries the *URA3* marker. The construct was used for transformation of diploid *Y. lipolytica* cells heterozygous for the *nukm*::*LEU2* allele, followed by sporulation and selection of haploids carrying both the *LEU2* marker of the *nukm*::*LEU2* allele and the *URA3* marker of the plasmid. Southern blot analysis of genomic DNA was carried out to demonstrate the absence of the chromosomal copy of *NUKM* in these strains. Single colonies were then grown overnight in YPD medium without selection for the *URA3* gene and then plated on YPD agar containing 5′-fluororotic acid (5′-FOA) to select for cells that had lost the plasmid-borne *URA3* marker. Under these conditions, loss of nonessential plasmids occurs with a frequency of 1×10^{-4} to 1×10^{-5} (Barth and Gaillardin, 1996). In our experiment, 5′-FOA resistant colonies appeared with approximately 10 times lower frequency. Southern blot analysis of ten colonies demonstrated that, although the *URA3* marker had been lost or damaged by spontaneous mutations in all of these strains, the plasmid-borne *NUKM* gene was still present. In some cases, deletions affecting the *URA3* marker extended far into the plasmid DNA, removing the ARS/CEN region (data not shown). These deletion products had apparently survived by integration into the *Y. lipolytica* genome. Failure to detect true plasmid loss among 5′-FOA resistant colonies indicated that complex I is not only essential for sporulation but also for vegetative growth. This is in stark contrast to NDH2, the external alternative NADH:ubiquinone oxidoreductase that had been shown previously to be a non-essential component (Kerscher et al., 1999).

Internal expression of NDH2

S. cerevisiae, which lacks respiratory chain complex I, in contrast to *Y. lipolytica* has alternative NADH:ubiquinone oxidoreductase activity both on the external (Luttik et al., 1998) and the internal (Marres et al., 1991) face of the mitochondrial inner membrane. We thus inferred that an internal alternative enzyme could be sufficient to carry out electron transfer from NADH to the respiratory chain ubiquinone pool in the absence of complex I. Plasmid pUB22 (Fig. 2), which contains the NDH2i gene encoding an internal version of the alternative NADH:ubiquinone oxidoreductase of *Y. lipolytica*, was created as described in Materials and Methods. The *NDH2i* gene was built by fusing the promoter region and the mitochondrial import signal of the 75 kDa subunit of complex I, encoded by the *NUAM* gene, to a truncated version of the *NDH2* open reading frame. Transformation of diploid Δ*nuam*/+ cells yielded between 200 and 2000 colonies per μg of pUB22 DNA, which is only 2-20% of the 1×10^3 to 1×10^4 colonies normally observed with replicative *Y. lipolytica* plasmids (Barth and Gaillardin, 1996). This may indicate that only a small fraction of the cells that had taken up the *HygB*^{R} gene were able to form colonies. Once established, however, transformants were stable. In contrast to the parental strain, transformants were able to grow on YPD agar containing 2 μM, 5 μM or 10 μM of the complex I inhibitor 2-decyl-4-quinazolinyl amine (DQA) (Okun et al., 1999a; Okun et al., 1999b), indicating that the *NDH2i* gene product could replace the NADH dehydrogenase function of complex I. Haploid *nuam*Δ, pUB22 strains were then generated by sporulation of diploid cells, followed by random spore analysis and selection of *URA3*, *HygB*^{R} colonies. In a similar fashion, haploid *nubm*Δ pUB22, *nucm*Δ pUB22, *nugm*Δ pUB22, *nuim*Δ pUB22 and *nukm*Δ pUB22 strains could be obtained. Such strains are valuable tools for the genetic dissection of complex I using the *Y. lipolytica* yeast genetic system.

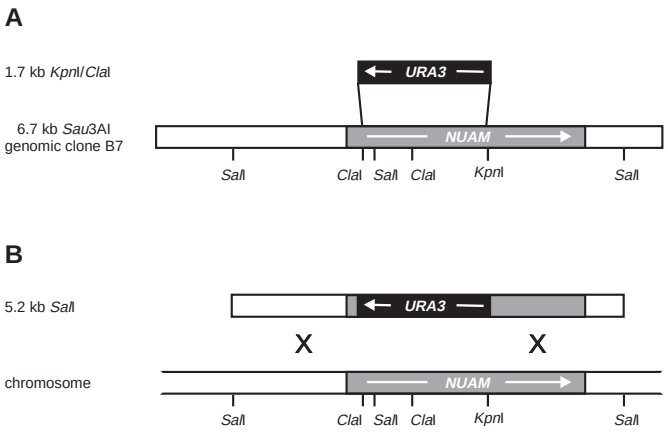
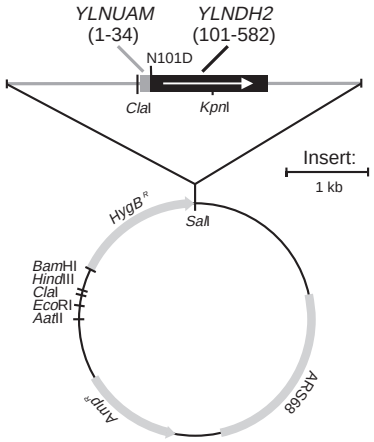


Fig. 1. Strategy for the deletion of the *Y. lipolytica NUAM* gene encoding the 75 kDa subunit of complex I. (A) Construction of the *nuam*::*URA3* deletion allele. (B) Deletion of one of the genomic *NUAM* copies in diploid GB1 cells by homologous recombination.

Mitochondrial membranes prepared from the *nuam*Δ pUB22 strain were analysed by blue-native polyacrylamide gel electrophoresis (BN-PAGE) (Schägger et al., 1994). The first



pUB 22

Fig. 2. Plasmid pUB22 for expression of the internal version of the alternative NADH:ubiquinone oxidoreductase of *Y. lipolytica*. Insert sequences derived from the *NDH2* gene locus are depicted in black, sequences derived from the *NUAM* gene locus are depicted in gray. Unique restriction sites are indicated.

native dimension showed that complex I was absent. Novel bands indicative of assembly intermediates could not be detected. Also, the second dimension (denaturing PAGE) gave no hint of protein bands derived from dissociated subcomplexes of complex I (data not shown).

To assess the DQA resistance conferred by plasmid pUB22 in more quantitative terms, GB5.2, a haploid *ndh2*Δ strain, was transformed with either the empty vector pUB4 or with plasmid pUB22 (Fig. 2). Transformants were cultivated in glucose-containing complete media under hygromycin B selection to a density of approximately 1×10^8 cells/ml. 1 μl each of dilutions containing 10, 100 or 1000 cells/ml were plated on glucose-containing complete media with increasing concentrations of the complex I inhibitor DQA (Fig. 3). Although all strains were able to grow in the presence of up to 0.25 μM DQA, only strains that possessed the *NDH2i* gene could survive in the presence of DQA concentrations ranging from 0.75 μM up to 10.0 μM. However, growth was significantly delayed at higher inhibitor concentrations (data not shown). It should be noted that strains in which complex I was present displayed the convoluted colony morphology typical for mycelial growth, while the *nuam*Δ pUB22 strain produced colonies that were predominantly smooth.

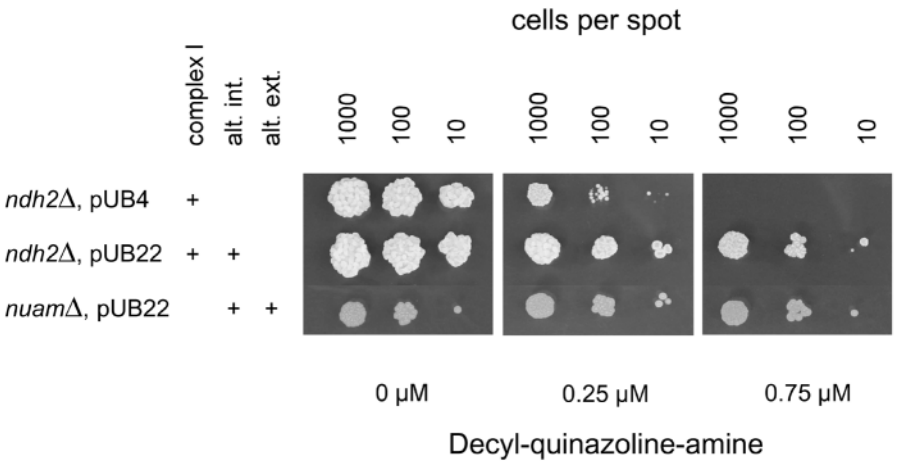


Fig. 3. Internal expression of alternative NADH:ubiquinone oxidoreductase allows survival in the presence of the complex I inhibitor DQA and survival of *nuam*Δ cells. See text for details.

Expression of other alternative NADH:ubiquinone oxidoreductases in *Y. lipolytica*

In parallel to the *YLNUAM-NDH2* fusion, we also generated the analogous fusion of the *NUAM* promoter with *NDH* from *E. coli* targeted with the *NUAM* mitochondrial import sequence. In another construct, the complete open reading frame of *NDI1* from *S. cerevisiae* retaining the *S. cerevisiae* targeting signal was placed behind the same strong hybrid promoter that was used to express the $HygB^R$ marker of plasmid pUB4.

Plasmid pUB10, which harbours the *YLNUAM-ECNDH* fusion, conferred some resistance to DQA. However, when compared with the *YLNUAM-NDH2* fusion, colony growth was slower and the survival rate of *ndh2*Δ pUB10 cells declined sharply at DQA concentrations of 1 μM or higher (Fig. 4). This may be explained by a low expression level of the *E. coli* enzyme, due to divergent codon usage. Consistent with this hypothesis, the *YLNUAM-ECNDH* fusion showed a clear gene dosage effect. Plasmid pUB11, which contains two gene copies of the *YLNUAM-ECNDH* fusion, resulted in markedly elevated DQA resistance (Fig. 4).

No resistance was observed with *Y. lipolytica* cells harbouring the *SCNDI1* gene contained in plasmid pUB28. The SCNDI1 protein was not imported into *Y. lipolytica* mitochondria, indicating that the *S. cerevisiae* targeting signal was not recognized. In a western blot probed with monoclonal antibody 22-2-B8, directed against SCNDI1 (de Vries and Grivell, 1988), the protein was detectable in intact mitochondria from *S. cerevisiae* KM91, but not from a *Y. lipolytica ndh2*Δ pUB4::*SCNDI1* strain, indicating that the fusion protein was degraded rather than imported (Fig. 5). This result was unexpected, since it has been shown that SCNDI1 can substitute for complex I in cultured mammalian cells and bears the promise of a genetic cure for hereditary complex I defects in humans (Seo et al., 1998; Seo et al., 1999). Inspection of the import signal sequences revealed that, while mitochondrial import of all seven central, nuclear coded subunits of complex I from *Y. lipolytica* entails processing at a site containing an arginine residue at position –3 (Djafarzadeh et al., 2000), the presequence of SCNDI1 has arginine at position –1 (de Vries et al., 1992). We thus speculated that the –1 arginine was not recognized by the matrix processing

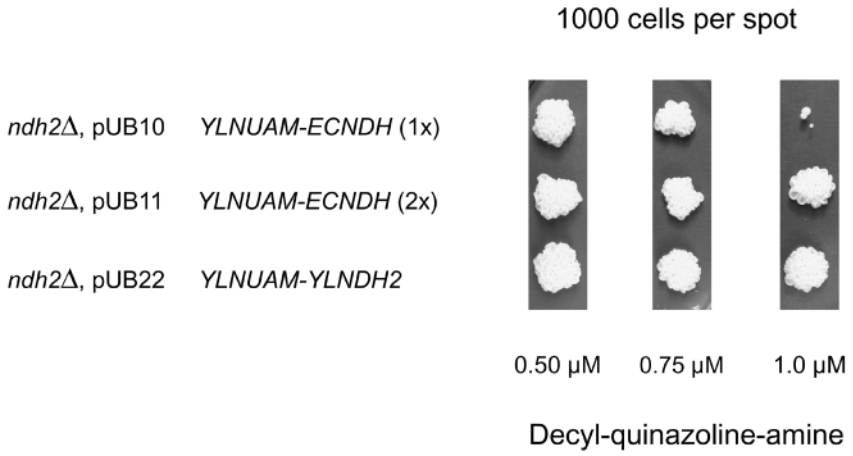


Fig. 4. DQA resistance conferred by the *YLNUAM-ECNDH* gene fusion. The gene dosage effect is evident from comparing the phenotypes of *ndh2*Δ pUB10 (one gene copy) and *ndh2*Δ pUB11 (two gene copies) cells. The phenotype of *ndh2*Δ pUB22 cells is included for comparison.

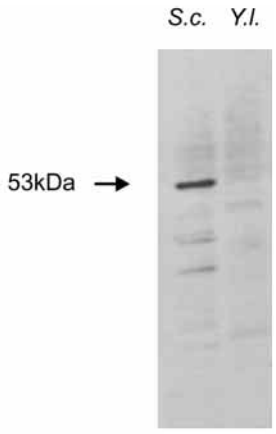


Fig. 5. SCNDI1 cannot be imported into *Y. lipolytica* mitochondria. On a western blot of intact mitochondria (1.5 μg of total protein) from *S. cerevisiae* KM91 (*S.c.*) and from *Y. lipolytica ndh2*::*URA3*, pUB4::*SCNDI1* (*Y.l.*) probed with α-SCNDI1 monoclonal antibody 22-2-B8, SCNDI1 is not detectable in *Y. lipolytica*.

peptidase of *Y. lipolytica* and that correct processing is required for a functional internal alternative NADH:ubiquinone oxidoreductase. To test this hypothesis, two alanine codons were introduced by site-directed mutagenesis behind the –1 arginine in SCNDI1 to create a '*Y. lipolytica* type' –3 arginine processing site. The resulting construct, termed pUB27, was again used for transformation of the *ndh2*Δ strain. Both constructs did not confer resistance to DQA (Fig. 6). Apparently, apart from the differences in the processing site, there are features in the SCNDI1 sequence that seem to preclude its import into *Y. lipolytica* mitochondria.

Metabolic effects of internal alternative NADH:ubiquinone oxidoreductase

To assay the effects of the internal alternative NADH:ubiquinone oxidoreductase and its interplay with respiratory chain complex I on cellular metabolism, we have determined the specific growth rates of *Y. lipolytica* strains possessing various sets of NADH:ubiquinone oxidoreductases in shake-flask cultures on complete media containing glucose or acetate as the carbon source. Strains carrying functional complex I and either the internal version of alternative NADH:ubiquinone oxidoreductase, or no alternative NADH:ubiquinone oxidoreductase activity at all were generated by transforming *ndh2*Δ strain GB5.2 with a $HygB^R$-marked single copy vector harbouring the gene for the internal version of NDH2 (pUB22), or with the empty vector pUB4 (Fig. 2). This strategy eliminates variations in the genetic background that cannot be fully excluded when using strains derived by sporulation. These strains were compared with the *nuam*Δ pUB22 strain in which complex I is absent and has been functionally replaced by the internal version of alternative NADH:ubiquinone oxidoreductase, NDH2i (Table 1).

On complete media containing glucose, strains that possessed complex I had very similar growth rates, irrespective of whether internal alternative NADH:ubiquinone oxidoreductase was present. By contrast, the specific growth rate of strain *nuam*Δ pUB22, which lacks complex I was reduced by about 10%. This may reflect the lower ATP yield that results from respiratory chain electron transport through the alternative enzyme which does not contribute to the transmembrane proton gradient.

On complete media containing acetate, the specific growth rate of the GB5.2 strain that lacked alternative NADH:ubiquinone oxidoreductase was reduced by about 20% compared with the strain that possessed the internal version of this enzyme. The specific growth rate of the *nuam*Δ pUB22 strain could not be assayed since no change in OD_{600} could be observed over 4 days. This strain did grow after incubation for 7 days, but then growth rates varied between different batches.

DISCUSSION

Y. lipolytica is an obligately aerobic yeast that possesses respiratory chain complex I and a single, external alternative NADH:ubiquinone oxidoreductase called NDH2. The alternative enzyme is not essential and the specific growth rate of the *ndh2*Δ strain was unaffected in complete media containing glucose or acetate (Kerscher et al., 1999). Studies in *S. cerevisiae* gave similar results. This fermentative yeast has two external alternative enzymes, called SCNDE1 and SCNDE2 (Luttik et al., 1998) and an internal alternative enzyme, called SCNDI1 (Marres et al., 1991). Deletion of *NDE1*, encoding the major external alternative enzyme of *S. cerevisiae*, or a double deletion of both *NDE1* and *NDE2* was still compatible with fully respiratory growth in glucose-limited chemostat cultures (Luttik et al., 1998; Small and McAlister-Henn, 1998). These data indicate that other pathways, such as the glycerol-3-phosphate dehydrogenase system (Larsson et al., 1998; Bjorkqvist et al., 1997; Overkamp et al., 2000), exist for the reoxidation of NADH generated in the cytoplasm.

Here we show that a strain that carries both complex I and a genetically engineered internal version of NDH2 displays a virtually identical growth rate on glucose, and an even higher growth rate on acetate when compared with the *ndh2*Δ strain. This may indicate either that the internal alternative enzyme does not efficiently compete with complex I for the substrates NADH and ubiquinone or that the reduction in ATP yield that results from the action of the alternative enzyme is not detrimental to the cell. An internal alternative enzyme may even be beneficial in preventing over-reduction of the mitochondrial matrix.

In sharp contrast to the mild phenotype of *ndh2*Δ strains, we

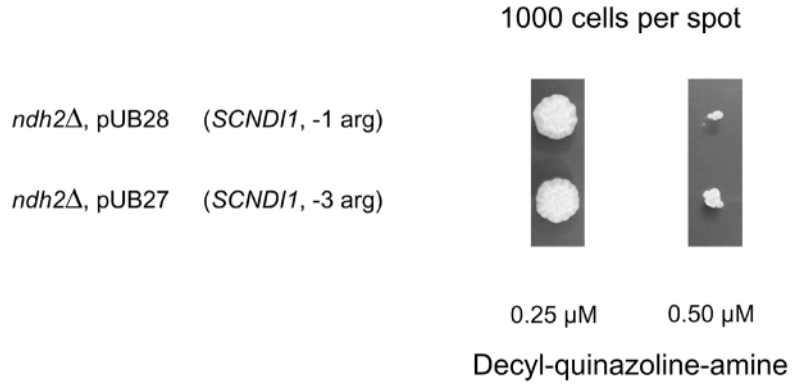


Fig. 6. SCNDI1 does not confer DQA resistance, either with the wild-type –1 arginine (pUB28) or the genetically engineered –3 arginine (pUB27) processing site.

Table 1. Specific growth rates (μ) of *Y. lipolytica* strains

Strain	NADH:ubiquinone oxidoreductase present*			Specific growth rate μ [hour^{-1}]‡	
	Complex I	Alt. int.	Alt. ext.	Glucose	Acetate
GB5.2, pUB4	+			0.324±0.003	0.115±0.000
GB5.2, pUB22	+	+		0.327±0.003	0.146±0.001
*nuam*Δ, pUB22		+	+	0.287±0.002	–

*Alt. int., alternative internal; Alt. ext., alternative external.
‡Values are the means of three independent experiments±s.e.m.

were unable to isolate haploid strains carrying deletions in genes for highly conserved subunits of complex I, proving that complex I is essential in *Y. lipolytica*. This result was surprising, since deletion of highly conserved subunits of complex I had previously been reported in other ascomycetous fungi such as *N. crassa* (Videira, 1998) and *Aspergillus niger* (Prömper et al., 1993). Survival of such mutant strains had been explained by postulating the presence of internal alternative NADH:ubiquinone oxidoreductase in these species, in analogy to *S. cerevisiae*, where respiratory chain complex I is absent (Büschges et al., 1994) but internal alternative NADH:ubiquinone oxidoreductase is present instead (de Vries and Grivell, 1988; Marres et al., 1991). Here we show that *Y. lipolytica* can survive without complex I only when NDH2 is redirected to the internal face of the mitochondrial inner membrane by the N-terminal attachment of the NUAM mitochondrial targeting signal.

This situation is remarkably different from that in *S. cerevisiae*, which lacks complex I and can even survive without SCNDI1 (Marres et al., 1991). *scndi1*Δ strains, which have no internal NADH:ubiquinone oxidoreductase at all, are still able to generate ATP by oxidative phosphorylation, as shown from their lack of a petite phenotype on fermentable substrates and their ability to grow on highly reduced, nonfermentable carbon sources such as ethanol (Marres et al., 1991).

The phenotype of *scndi1*Δ strains had initially been explained by claiming that with highly reduced substrates such as glucose or ethanol, NADH production in the cytoplasm and transfer of redox equivalents to the mitochondrial ubiquinone pool by external alternative NADH:ubiquinone oxidoreductase or alternative pathways is sufficient to permit ATP synthesis by oxidative phosphorylation (Marres et al., 1991). However, recent findings indicate that survival of the *scndi1*Δ mutant depends on NADH generated in the mitochondrial matrix and transferred into the cytoplasm by the ethanol-acetaldehyde shuttle (Bakker et al., 2000). Unlike most of the well-studied shuttles for the transfer of redox equivalents into the mitochondrial matrix, which involve active transport steps (Dawson, 1979), this shuttle is reversible (von Jagow and Klingenberg, 1970; Bakker et al., 2000). A double mutant strain carrying deletions in both *SCNDI1* and *ADH3*, the gene encoding mitochondrial alcohol dehydrogenase, exhibited a reduction in biomass yield on glucose and increases in ethanol production and respiratory quotient, indicative of respirofermentative metabolism in aerobic, glucose-limited chemostat cultures. By contrast, both single deletion strains exhibited fully respiratory growth under the same conditions (Bakker et al., 2000). However, *scndi1*Δ strains cannot grow on highly oxidised, nonfermentable substrates such as acetate (Marres et al., 1991). Since it may be argued that during growth on acetate NADH produced in the citric acid cycle could be transferred into the cytoplasm via the ethanol-acetaldehyde shuttle and could then be fed into the respiratory chain, this finding clearly demonstrates that the metabolic capacity of this shuttle system is limited.

The enzymes necessary for an ethanol-acetaldehyde shuttle may be present in *Y. lipolytica*. Three genes for alcohol dehydrogenases (GenBank accession nos AAD51737, AAD51738 and AAD51739) have been identified in this species. Pyruvate decarboxylase is encoded in the genomes of several yeasts, including non-fermentative species. Three STS sequences (GenBank accession nos AL411453, AL412002 and AL412286) for two *Y. lipolytica* homologs of *S. cerevisiae* pyruvate decarboxylase were identified as part of a random genomic sequencing program of 13 yeast species (Casaregola et al., 2000; Souciet et al., 2000). If an ethanol-acetaldehyde shuttle does operate in *Y. lipolytica*, its efficiency must be lower than in *S. cerevisiae.* Insufficient redox shuttle activity would explain why *Y. lipolytica* strains that, owing to a deletion in one of the genes for the central subunits of complex I, lack all internal NADH:ubiquinone oxidoreductase activity were unable to survive even on complete media containing highly reduced carbon sources such as glucose.

The results presented here also shed new light on the mitochondrial import of NDH2. The fact that NDH2i could substitute for complex I demonstrates that the internalised enzyme does associate with the inner face of the mitochondrial inner membrane and is fully capable of interacting with the ubiquinone pool of the respiratory chain. As *Y. lipolytica* does not normally contain an internal alternative enzyme, this strongly suggests that membrane association does not require interaction with any protein component in the mitochondrial inner membrane. Also, integration of the enzyme's redox prosthetic group, a non-covalently bound molecule of FAD, has to take place either before mitochondrial import or must be possible in a similar way both in the cytosol and in the mitochondrial matrix.

Successful redirection of NDH2 just by attaching a mitochondrial targeting signal supports the notion that the association of alternative NADH:ubiquinone oxidoreductases with the internal face of the mitochondrial inner membrane was brought about by one single evolutionary step following gene duplication, namely the acquisition of a matrix targeting sequence by an originally external alternative enzyme.

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REFERENCES

Ahlers, P. M., Garofano, A., Kerscher, S. and Brandt, U. (2000a). Application of the obligate aerobic yeast *Yarrowia lipolytica* as a eucaryotic model to analyze Leigh Syndrome mutations in the complex I core subunits PSST and TYKY. *Biochim. Biophys. Acta* **1459**, 258-265.

Ahlers, P. M., Zwicker, K., Kerscher, S. and Brandt, U. (2000b). Function of conserved acidic residues in the PSST-homologue of complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica*. *J. Biol. Chem.* **275**, 23577-23582.

Bakker, B., 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.

Barth, G. and Gaillardin, C. (1996). *Yarrowia lipolytica*. In *Non-Conventional Yeasts in Biotechnology*. (ed. K. Wolf), pp. 313-388. Berlin-Heidelberg: Springer Verlag.

Bjorkqvist, S., Ansell, R., Adler, L. and Liden, G. (1997). Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **63**, 128-132.

Brandt, U. (1999). Proton translocation in the respiratory chain involving ubiquinone - A hypothetical semiquinone switch mechanism for complex I. *BioFactors* **9**, 95-101.

Büschges, R., Bahrenberg, G., Zimmermann, M. and Wolf, K. (1994). NADH:ubiquinone oxidoreductase in obligate aerobic yeasts. *Yeast* **10**, 475-479.

Casaregola, S., Neuveglise, C., Lepingle, A., Bon, E., Feynerol, C., Artiguenave, F., Wincker, P. and Gaillardin, C. (2000). Genomic exploration of the hemiascomycetous yeasts: 17. *Yarrowia lipolytica*. *FEBS Lett.* **487**, 95-100.

de Vries, S. and Grivell, L. A. (1988). Purification and characterization of a rotenone-insensitive NADH:Q6 oxidoreductase from mitochondria of *Saccharomyces cerevisiae. Eur. J. Biochem*. **176**, 377-384.

de Vries, S., van Witzenburg, R., Grivell, L. A. and Marres, C. A. M. (1992). Primary structure and import pathway of the rotenone-insensitive NADH-ubiquinone oxidoreductase of mitochondria from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **203**, 587-592.

Dawson, A. G. (1979). Oxidation of cytosolic NADH formed during aerobic metabolism in mammalian cells. *Trends Biochem. Sci*. **4**, 171-176.

Djafarzadeh, R., Kerscher, S., Zwicker, K., Radermacher, M., Lindahl, M., Schägger, H. and Brandt, U. (2000). Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*. *Biochim. Biophys. Acta* **1459**, 230-238.

Finel, M. (1998). Does NADH play a central role in energy metabolism in *Helicobacter pylori*? *Trends Biochem. Sci*. **23**, 412-414.

Gritz, L. and Davies, J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**, 179-188.

Kashani-Poor, N., Kerscher, S., Zickermann, V. and Brandt, U. (2001). Efficient large scale purification of his-tagged proton translocating NADH:ubiquinone oxidoreductase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*. *Biochim. Biophys. Acta* **1504**, 363-370.

Kerscher, S. (2000). Diversity and origin of alternative NADH:ubiquinone oxidoreductases. *Biochim. Biophys. Acta* **1459**, 274-283.

Kerscher, S., Okun, J. G. and Brandt, U. (1999). A single external enzyme confers alternative NADH:ubiquinone oxidoreductase activity in *Yarrowia lipolytica*. *J. Cell Sci.* **112**, 2347-2354.

Lagunas, R. (1986). Misconceptions about the energy metabolism of *Saccharomyces cerevisiae*. *Yeast* **2**, 221-228.

Larsson, C., Pahlman, I. L., Ansell, R., Rigoulet, M., Adler, L. and Gustafsson L. (1998). The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. *Yeast* **14**, 347-357.

Luttik, M. A. H., Overkamp, K. M., Kötter, P., de Vries, S., van Dijken, 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.* **273**, 24529-24534.

Madzak, C., Blanchin-Roland, S., Otero, R. C. and Gaillardin, C. (1999). Functional analysis of upstream regulating regions from the *Yarrowia lipolytica* XPR2 promoter. *Microbiology* **145**, 75-87.

Madzak, C., Treton, B. and Blanchin-Roland S. (2000). Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J. Mol. Microbiol. Biotechnol.* **2**, 207-216.

Marres, C. A. M., 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.

Ohnishi, T. (1998). Iron-sulfur clusters semiquinones in complex I. *Biochim. Biophys. Acta* **1364**, 186-206.

Okun, J. G., Lümmen, P. and Brandt, U. (1999a). Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase). *J. Biol. Chem.* **274**, 2625-2630.

Okun, J. G., Zickermann, V. and Brandt, U. (1999b). Properties of the common inhibitor binding domain in mitochondrial NADH-dehydrogenase (complex I). *Biochem. Soc. Trans.* **27**, 596-601.

Okun, J. G., Zickermann, V., Zwicker, K., Schägger, H. and Brandt, U. (2000). Binding of detergents and inhibitors to bovine complex I - A novel purification procedure of bovine complex I retaining full inhibitor sensitivity. *Biochim. Biophys. Acta* **1459**, 77-87.

Overkamp, K. M., Bakker, B., Kötter, P., van Tuijl, A., de Vries, S., van Dijken, J. P. and Pronk, J. T. (2000). In vivo analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria. *J. Bacteriol.* **182**, 2823-2830.

Prömper, C., Schneider, R. and Weiss, H. (1993). The role of the proton-pumping and alternative respiratory chain NADH:ubiquinone oxidoreductases in overflow catabolism of *Aspergillus niger*. *Eur. J. Biochem.* **216**, 223-230.

Sazanov, L. A., Peak-Chew, S. Y., Fearnley, I. M. and Walker, J. E. (2000). Resolution of the membrane domain of bovine complex I into subcomplexes: Implications for the structural organization of the enzyme. *Biochemistry* **39**, 7229-7235.

Schägger, H., Cramer, W. A. and von Jagow, G. (1994). Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**, 220-230.

Seo, B. B., Kitajima-Ihara, T., Chan, E. K. L., Scheffler, I. E., Matsuno-Yagi, A. and Yagi, T. (1998). Molecular remedy of complex I defects: Rotenone-insensitive internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells. *Proc. Natl. Acad. Sci. USA* **95**, 9167-9171.

Seo, B. B., Matsuno-Yagi, A. and Yagi, T. (1999). Modulation of oxidative phosphorylation of human kidney 293 cells by transfection with the internal rotenone-insensitive NADH-quinone oxidoreductase (*NDI1*) gene of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1412**, 56-65.

Small, W. C. and McAlister-Henn, L. (1998). Identification of a cytosolically directed NADH dehydrogenase in mitochondria of *Saccharomyces cerevisiae*. *J. Bacteriol.* **180**, 4051-4055.

Souciet, J. L., Aigle, M., Artiguenave, F., Blandin, G., Bolotin-Fukuhara, M., Bon, E., Brottier, P., Casaregola, S., de-Montigny, J., Dujon, B. et al. (2000). Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies. *FEBS Lett.* **487**, 3-12.

Videira, A. (1998). Complex I from the fungus *Neurospora crassa*. *Biochim. Biophys. Acta* **1364**, 89-100.

von Jagow, G. and Klingenberg, M. E. (1970). Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. *Eur. J. Biochem.* **12**, 583-592.

Walker, J. E. (1992). The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. *Q. Rev. Biophys.* **25**, 253-324.

Young, I. G., Rogers, B. L., Campbell, H. D., Jaworowski, A. and Shaw, D. C. (1981). Nucleotide sequence coding for the respiratory NADH dehydrogenase of *Escherichia coli*. UUG initiation codon. *Eur. J. Biochem.* **116**, 165-170.