NAD\(^+\)-dependent Isocitrate Dehydrogenase

CLONING, NUCLEOTIDE SEQUENCE, AND DISRUPTION OF THE IDH2 GENE FROM SACCHAROMYCES CEREVISIAE*

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NAD\(^+\)-dependent isocitrate dehydrogenase from *Saccharomyces cerevisiae* is composed of two nonidentical subunits, designated IDH1 (Mr, 40,000) and IDH2 (Mr, 39,000). We have isolated and characterized a yeast genomic clone containing the IDH2 gene. The amino acid sequence deduced from the gene indicates that IDH2 is synthesized as a precursor of 369 amino acids (Mr, 39,694) and is processed upon mitochondrial import to yield a mature protein of 354 amino acids (Mr, 37,755). Amino acid sequence comparison between *S. cerevisiae* IDH2 and *S. cerevisiae* NAD\(^+\)-dependent isocitrate dehydrogenase shows no significant sequence identity, whereas comparison of IDH2 and *Escherichia coli* NAD\(^+\)-dependent isocitrate dehydrogenase reveals a 33% sequence identity.

To confirm the identity of the IDH2 gene and examine the relationship between IDH1 and IDH2, the *IDH2* gene was disrupted by genomic replacement in a haploid yeast strain. The disruption strain expressed no detectable IDH2, as determined by Western blot analysis, and was found to lack NAD\(^+\)-dependent isocitrate dehydrogenase activity, indicating that IDH2 is essential for a functional enzyme. Overexpression of IDH2, however, did not result in increased NAD\(^+\)-dependent isocitrate dehydrogenase activity, suggesting that both IDH1 and IDH2 subunits are required for catalytic activity. The disruption strain was unable to utilize acetate as a carbon source and exhibited a 2-fold slower growth rate than wild type strains on glyceral or lactate. This growth phenotype is consistent with NAD\(^+\)-dependent isocitrate dehydrogenase performing an essential role in the oxidative function of the citric acid cycle.

Isocitrate dehydrogenase catalyzes a rate-limiting step of the citric acid cycle, the conversion of isocitrate to a-ketoglutarate coupled to the production of NADH. Mitochondrial NAD\(^+\)-dependent isocitrate dehydrogenase (NAD\(^+\)-IDH)\(^1\) is present in all eukaryotic cells and has been purified from a variety of sources, including *Saccharomyces cerevisiae* (1), *Neurospora crassa* (2), and pig (3) and bovine (4) heart. Characterization of purified NAD\(^+\)-IDH has shown it to be a complex oligomeric enzyme that is subject to extensive allosteric regulation. The enzyme from *S. cerevisiae* functions as an octamer composed of two nonidentical subunits, designated IDH1 (Mr, 40,000) and IDH2 (Mr, 39,000) (6), and is responsive to cellular energy levels as a result of binding and activation by AMP and NAD\(^+\) (6).

The roles of the individual subunits of NAD\(^+\)-IDH in catalysis and regulation are not yet understood. The number of binding sites within NAD\(^+\)-IDH from *S. cerevisiae* for the allosteric regulator AMP, the substrate isocitrate, and the cofactor NAD\(^+\) was determined using equilibrium dialysis to be fewer than the number of subunits for each compound (7). Similar results have been reported for the pig (8) and bovine (9) NAD\(^+\)-IDH enzymes. Two models of subunit function and interaction can account for the results of these studies. The individual IDH1 and IDH2 subunits may have a specialized function in catalysis or regulation, with each containing complete, independent binding sites; alternatively, the subunits may contain half-binding sites with interaction between IDH1 and IDH2 required to form a complete binding pocket. Determination of the contribution of each subunit to the function and regulation of NAD\(^+\)-IDH will provide insight into the control of substrate flow through the citric acid cycle.

In addition to mitochondrial NAD\(^+\)-IDH, *S. cerevisiae* contains cytoplasmic and mitochondrial isocitrate dehydrogenase enzymes that utilize NADP\(^+\) as a cofactor. The two mitochondrial isocitrate dehydrogenase isozymes are differentially expressed in response to carbon source and oxygen levels (10), suggesting that they function in separate pathways. The metabolic function of the NADP\(^+\)-dependent isocitrate dehydrogenase (NADP\(^+\)-IDH) isozyme has not been determined, but they are presumed to play a role in biosynthetic reactions that require NADP(H) and/or a-ketoglutarate. The gene encoding the mitochondrial NADP\(^+\)-IDH has recently been cloned, and genomic disruption of the gene (11) did not result in the acetate-growth phenotype characteristic of yeast citric acid cycle mutants (12, 13). These results suggest that the mitochondrial NADP\(^+\)-IDH is not the primary isozyme of isocitrate dehydrogenase that functions in the citric acid cycle. The NADP\(^+\)-IDH isozymes may, however, under certain conditions have compensatory roles in the citric acid cycle. Further analysis of the metabolic function of each isozyme will require the development of yeast strains lacking one or more of the isocitrate dehydrogenase isozymes.

We present in this paper the cloning and nucleotide sequence of the gene encoding the IDH2 subunit of *S. cerevisiae* NAD\(^+\)-IDH. A strain lacking IDH2 was constructed by one-step genomic replacement. The disruption strain was characterized by lack of NAD\(^+\)-IDH activity *in vitro* and by altered growth rates on a variety of carbon sources. Additionally,
amino acid sequence comparison of the yeast IDH2 subunit and Escherichia coli NADP^*--IDH revealed similarities between the eukaryotic NAD^*--IDH enzyme and the prokaryotic NADP^*--IDH enzyme.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—Yeast strains lacking a functional NAD^*--IDH were previously identified (5) from screens of mutants that failed to grow on acetate as a carbon source (provided by Dr. M. McCammon, University of Texas Southwestern Medical Center, Dallas). Complementation experiments were performed using the NAD^*--IDH mutants designated idh1-1 and idh2-1. The alleles were previously characterized and shown to lack the 40,000 and 39,000 molecular weight subunits, respectively (5). The ΔIDH2 strain was constructed using the wild type haploid yeast strain S173–6B (MATa leu2-3,112 his3-1 ura3-52 trpl–289). Yeast strains were grown in rich YP medium (1% yeast extract, 2% Bacto-Peptone (Difco), semisynthetic NYN/RE medium (0.17% nitrogen base, 0.5% ammonium sulfate (Difco), 0.05% yeast extract, pH 6.0), or minimal YNB medium (0.17% nitrogen base (Difco), 0.5% ammonium sulfate, pH 6.0). Minimal medium contained supplements of 20 mg/ml to satisfy auxotrophic requirements for growth. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of reagent grade. Yeast strains were propagated in a water-jacketed shaking incubator at 30°C.

Cell Fractionation—Mitochondrial extracts for use in enzyme assays and Western blot analysis were obtained by vortexing yeast cells with glass beads followed by centrifugation in a microcentrifuge to remove membranes. Protein concentrations were determined using the Bradford dye-binding method (22). Western Blot Analysis—Samples of mitochondrial protein extracts (20 μg each) for Western blot analysis were electrophoresed on 10% polyacrylamide–sodium dodecyl sulfate gels (23). Separated proteins were transferred to polyvinylidene membranes (Immobilon, Millipore Corp.) using a graphite dry blotting apparatus (24). The membrane was stained with Coomassie Blue, blocked with 5% bovine serum albumin, and incubated in a 1:300 dilution of anti-IDH antisera (5). Detection was performed using 125I-labeled protein A following autoradiography.

RESULTS

Cloning and Nucleotide Sequencing—As a strategy to isolate the IDH1 and IDH2 genes, yeast strains lacking functional NAD^*--IDH, idh1-1 and idh2-1 (5), were used for complementation. The IDH^* strains were transformed with a yeast genomic DNA library containing 5–15-kb fragments of yeast genomic DNA cloned into the shuttle vector YCP50. This vector has a single copy Cen origin of replication and a yeast URA3 gene for selection. Transformants were initially selected on minimal (YNB) medium plus glucose plates that lacked uracil to determine transformation efficiency. Transformants were collected and replated on rich (YP) acetate for selection of plasmids that would complement the IDH^* phenotype, a failure to grow with acetate as a carbon source. A single complementing plasmid was isolated from one of more than 10,000 independent transformants and was found to contain an 8-kb yeast genomic DNA insert. The gene complementing the IDH^* defect was localized to a 2.8-kb EcoRI-HindIII fragment by Southern blot analysis using a degenerate oligonucleotide probe encoding the amino terminus of IDH2, as described under "Materials and Methods." A partial restriction map of the 2.8-kb EcoRI-HindIII fragment and the strategy used for nucleotide sequencing of this region are shown in Fig. 1.

Nucleotide sequence analysis revealed an open reading frame (Fig. 2) that encodes the amino terminus of the mature IDH2 polypeptide (nucleotides 46–89), which was previously determined by amino acid sequence analysis (5). An ATG codon located 45 nucleotides upstream from the sequence encoding the mature form of IDH2 is presumed to be the initiator methionine codon, because it is the first methionine codon in the identified open reading frame and is preceded by a potential TATA box at relative nucleotide positions –13 to –17. Assuming that translation begins at this methionine codon, the deduced amino acid sequence predicts a precursor protein of 309 amino acids (M, 39,894); cleavage of a 15-residue amino-terminal peptide upon mitochondrial import yields the mature protein of 354 amino acids (M, 37,758). The presumptive mitochondrial targeting peptide is similar in size and properties to other previously characterized yeast mitochondrial import peptides (25, 26).

IDH2 Gene Disruption—To confirm the identity of the IDH2 gene and to examine the relationship between IDH2 and IDH1, the majority of the coding region of the cloned gene was replaced with the yeast HIS3 gene and used for one-step genomic replacement (27). Fig. 3A illustrates the deletion-insertion construct in which a 1.8-kb fragment containing the coding region and adjacent 3'–noncoding region was replaced by a 1.7-kb fragment containing the yeast HIS3 gene. Genomic recombination in a His^* haploid yeast strain was achieved by transformation with a linear 3.0-kb EcoRI-EcoRV fragment (cf. Fig. 1) containing the HIS3 disruption. To confirm integration at the IDH2 locus, Southern blot analysis
was performed on genomic DNA isolated from the wild type and disruption strains that had been digested with EcoRI plus EcoRV. As shown in Fig. 3B for DNA from the wild type strain, two bands with expected sizes of approximately 1.1 and 1.9 kb hybridized with a 32P-labeled IDH2 probe (the 2.8-kb EcoRI-HindIII DNA fragment). In DNA from the disruption strain, however, a single 3.0-kb fragment hybridized to the gene. To confirm that the observed alteration was due to recombination of the disruption strains that had been digested with EcoRI-HindIII, a Southern blot of the same DNA digests was hybridized with a 32P-labeled HIs3 probe. The endogenous HIs3 gene was detected as a 5.0-kb fragment in DNA from both the wild type and the disruption strains. An additional 3.0-kb fragment, corresponding to the 2.8-kb fragment that has been detected by the 32P-labeled IDH2 probe, was present in DNA from the disruption strain, indicating integration of the HIs3 gene at the IDH2 locus.

The absence of expression of the IDH2 subunit in the disruption (∆IDH2) strain was confirmed by Western blot analysis (Fig. 4). Using an anti-IDH antisera prepared against the purified yeast holoenzyme (5), bands corresponding to both the IDH1 (M, ~40,000) and IDH2 (M, ~39,000)
Expression of NAD+-dependent isocitrate dehydrogenase in wild type and \( \Delta IDH2 \) strains. Immunoblot analysis of NAD+-dependent isocitrate dehydrogenase was conducted as described under "Material and Methods." Genomic integration was achieved by transformation using a linear 3.0-kb EcoRI-EcoRV fragment containing the \( IDHZ \) disruption. A, Southern blot analysis of DNA from the wild type strain and the \( \Delta IDH2 \) strain. Aliquots (10 \( \mu \)g) of total genomic DNA isolated from wild type (lanes 1) and \( \Delta IDH2 \) (lanes 2) were digested with EcoRI and EcoRV and subjected to electrophoresis. Southern blot analysis was performed using an \( ^{32}P \)-labeled probe the 2.8-kb EcoRI-HindIII DNA fragment from \( IDH2 \) (left panel) or a 1.7-kb BamHI fragment containing the \( HIS3 \) gene (right panel) as described in the text.

Subunit proteins were detected in mitochondrial extracts from the wild type strain (lane 1), whereas mitochondrial extracts from the \( \Delta IDH2 \) strain contained a single cross-reacting protein of \( M_r \approx 40,000 \) (IDH1) (lane 2). This result provides further evidence that the gene cloned encodes IDH2 and also establishes that the IDH1 subunit is encoded at a separate genomic locus.

Previous reports have shown that expression of NAD+-IDH is highly regulated and that approximately equivalent levels of the IDH1 and IDH2 subunits are maintained in wild type cells. As shown in Fig. 4, lane 2, disruption of \( IDH2 \), however, does not appear to alter levels of IDH1. This suggests that expression of the two subunits may not be coordinately controlled. To further examine expression of IDH1 and IDH2, single or multicopy shuttle vectors containing the \( IDH2 \) gene were transformed into the \( \Delta IDH2 \) strain, and levels of the subunits were examined by Western blot analyses as described in the Western blot analyses. As shown in Fig. 4, lane 3, expression of IDH2 using the single copy vector YCp50 resulted in wild type levels of IDH2 and a ratio of IDH1:IDH2 approximating 1:1. Overexpression of IDH2 using the multicopy vector YEp352, on the other hand, resulted in approximately a 30-fold increase, as determined by densitometry, in the levels of IDH2 as compared with IDH1 (Fig. 4, lane 4). These results suggest that the \( IDH1 \) and \( IDH2 \) genes are independently transcribed and that subunit protein levels do not regulate expression.

Activity and Growth Phenotype of the Disruption Strain—To determine if IDH1 or IDH2 can independently catalyze the conversion of isocitrate to \( \alpha \)-ketoglutarate, mitochondrial extracts from strains expressing different levels of IDH2 were assayed for NAD+-IDH activity (Table I). The \( \Delta IDH2 \) strain contained no detectable NAD+-IDH activity; this lack of activity was not due to an inhibitory factor in the \( \Delta IDH2 \) extract, because assays containing a mixture of wild type extract plus \( \Delta IDH2 \) extract exhibited full activity. These results indicate that IDH1 cannot independently catalyze the conversion of isocitrate to \( \alpha \)-ketoglutarate and that IDH2 is essential for NAD+-IDH activity. Overexpression of IDH2, on the other hand, using the multicopy vector YEp352 did not result in elevated NAD+-IDH activity, even though levels of IDH2 are approximately 30-fold higher in these extracts. This result suggests that, like IDH1, IDH2 alone does not exhibit NAD+-IDH activity. Together, these data indicate that both subunits are required for a functional enzyme. An attempt to reconstitute the NAD+-IDH activity in vitro by combining IDH1 in \( \Delta IDH2 \) extracts with IDH2 in \( \Delta IDH2 \) (YEp352-IDH2) extracts was unsuccessful. Although this was not extensively studied, formation of the active oligomeric form of the enzyme may require conditions or other cellular components that were absent in these assays.

The metabolic function of NAD+-IDH was examined by growth phenotype analysis of the \( \Delta IDH2 \) strain. Because the original selection used for yeast strains lacking NAD+-IDH activity was an inability to grow on semisynthetic medium with 2% sodium acetate as the carbon source, we characterized Table 1

| Strain (plasmid) | Specific activity | % of wild type |
|------------------|------------------|---------------|
| Wild type        | 100              |               |
| \( \Delta IDH2 \) | 85               |               |
| \( \Delta IDH2(YCp50-IDH2) \) | 89 |               |
| \( \Delta IDH2(YEp352-IDH2) \) | 89 |               |
| Wild type plus \( \Delta IDH2 \) | 116 |               |
| \( \Delta IDH2(YEp352-IDH2) \) plus \( \Delta IDH2 \) | 89 |               |

\( ^{a} \) ND, no detectable activity.

\( ^{b} \) \( IDH2 \) deletion strain containing the plasmid indicated in parentheses.

\( ^{c} \) Mixed assay in which the two extracts were combined and allowed to equilibrate at room temperature for 5 min prior to assay.
the growth phenotypes of the ∆IDH2 strain using acetate and other nonfermentable carbon sources. As shown in Table II, the ∆IDH2 strain grew at reduced rates on all nonfermentable carbon sources and exhibited wild type growth rates only on glucose (rich or semisynthetic medium). Growth rates for the ∆IDH2 strain on glycerol or lactate were reduced 2.5-fold in rich media and 1.5-fold in semisynthetic media as compared with the wild type strain. The difference between growth on nonfermentable carbon sources in rich medium versus semisynthetic medium may be due to increased expression of other enzymes under conditions of limiting nitrogen that provide compensation for the disruption defect. The ∆IDH2 strain doubled no more than once within a 48-h period following dilution from a rich (YP) glucose starter culture into rich or semisynthetic acetate medium. Expression of IDH2 using shuttle vectors in the ∆IDH2 strain was found to restore near wild type growth rates on all carbon sources, confirming that the metabolic defect is due to a loss of NAD⁺-IDH activity. A similar acetate⁻ phenotype has been reported for yeast strains containing defects in the citric acid cycle isozymes of citrate synthase (12) or mitochondrial malate dehydrogenase (13). Thus, the observed growth phenotype for the ∆IDH2 strain is consistent with earlier suggestions (5) that the isocitrate dehydrogenase that utilizes NAD⁺ is the isozyme that functions in the citric acid cycle.

**DISCUSSION**

**Subunit Interactions and Function—**We present here the first reported cloning and nucleotide sequence of a gene encoding an NAD⁺-dependent isocitrate dehydrogenase. Prior to this work, the only gene for an isocitrate dehydrogenase known to function in the citric acid cycle that had been cloned was that encoding the NAD⁺-dependent isocitrate dehydrogenase from *E. coli* (28). The NAD⁺-IDH enzyme from *S. cerevisiae*, however, is distinctly different from the *E. coli* enzyme. In addition to different cofactor specificities, the yeast enzyme exists as an octamer of two nonidentical subunits (29), whereas NAD⁺-IDH from *E. coli* exists as a dimer of identical subunits (28). Moreover, the enzyme from *E. coli* is regulated solely by phosphorylation (30), whereas the yeast enzyme is allosterically regulated by several compounds including AMP, NAD⁺, and citrate (6). The functional significance of a complex oligomeric NAD⁺-IDH enzyme in eukaryotic cells has been the subject of much speculation. Because the E. coli enzyme is not subject to allosteric regulation, it is possible that the eukaryotic NAD⁺-IDH enzyme in parallel with compartmentation of the citric acid cycle has evolved as a more complex oligomeric enzyme to allow for allosteric regulation.

Based on the studies that indicate four isocitrate- and two AMP- and NAD⁺-binding sites per octamer, it has been suggested that the individual subunits of yeast NAD⁺-IDH have separate functions of catalysis or regulation (6). Sequence differences among tryptic peptides from the individual subunits of pig heart NAD⁺-IDH have also been interpreted to suggest possible differences in functions of the subunits (31). Our findings with the disruption of the IDH2 gene, however, do not provide support for a model of independent subunit function in which one subunit is catalytically active in the absence of a regulatory subunit. Strains expressing only IDH1 showed no detectable NAD⁺-IDH activity, indicating that IDH1 in the absence of IDH2 is not catalytically active. Additionally, overexpression of IDH2 in the presence of wild type levels of IDH1 did not yield an increase in activity, suggesting that IDH2 can not function independently of IDH1. These results imply that both IDH1 and IDH2 are required for catalysis and suggest two possible models of subunit interaction and function. In one model, amino acid residues required for product formation, including either the substrate-binding site or catalytic site, are shared between IDH1 and IDH2; this is similar to the *E. coli* enzyme in which each subunit contains half-sites for isocitrate binding which interact with one another to form a complete binding site (32). Alternatively, the amino acid residues required for product formation may be localized on a single subunit, but interactions with the regulatory subunit would be required for catalysis. Determination of the correct model will likely require sequence analysis of the IDH1 gene, structural studies of the active octamer and isolated subunits, and alteration of the IDH2 and IDH1 genes by site-specific mutagenesis.

Although the precise function of each subunit can not be determined at this time, our results provide evidence that the two subunits of the yeast enzyme are not a result of post-translational modification of a single gene product. Deletion of the IDH2 gene does not alter expression of IDH1, confirming that IDH1 and IDH2 are encoded by separate genes. Additionally, overexpression of IDH2 using the multicopy shuttle vector YEp352 did not result in increased levels of IDH1. This result indicates that expression of one gene does not affect expression of the other.

**IDH2 Sequence Comparisons—**Despite the differences in cofactor specificity, subunit structure, and regulation between the yeast NAD⁺-IDH and *E. coli* NAD⁺-IDH enzymes, IDH2 and the prokaryotic polypeptide exhibit considerable similarity in amino acid sequence. The alignment shown in Fig. 5 yields an overall sequence identity of 33%, and two regions, residues 82–115 and 268–316 of IDH2, contain greater than 55% identity. Structural analysis of the *E. coli* enzyme by x-ray crystallography has established that residues 110–120, corresponding to residues 95–105 in IDH2, function in isocitrate binding (32). Ser-113 of the *E. coli* enzyme forms a hydrogen bond with isocitrate (33), and phosphorylation at this site inhibits binding of isocitrate (34). Ser-98 of yeast IDH2 aligns with this residue and may participate in substrate binding in the yeast NAD⁺-IDH octamer. Additional residues (Arg-114, Arg-129, Arg-153, Tyr-160, and Lys-230), which are near the binding site of isocitrate in the three-dimensional structure of the *E. coli* enzyme (32), are also conserved in IDH2 (Arg-104, Arg-114, Arg-135, Tyr-142, and Lys-189). The second region of high sequence similarity, amino acids 268–

**Table II**

| Growth rates (doubling time) | Strain | Rich medium | Semisynthetic medium |
|------------------------------|--------|-------------|----------------------|
|                              | Glc    | G11 | Lac | Ace | Glc | G11 | Lac | Ace |
| ∆IDH2                        | 1.8    | 3.1 | 3.9 | 5.1 | 2.2 | 3.2 | 3.7 | 5.1 |
| ∆IDH2(YEp50-IDH1)³           | 1.9    | 7.8 | 9.9 | NG⁴ | 2.4 | 5.3 | 5.6 | NG⁴ |
| ∆IDH2(YEp352-IDH2)⁵          | 1.8    | 3.3 | 4.0 | 5.3 |     |     |     |     |

*NG, no growth, indicating the inability to double more than once during a 48-h period.

IDH2 disruption strain containing the plasmid indicated in parentheses.
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... subunit from the limited data available. One region of sequence similarity of particular significance, however, is the region encompassing IDH2 residues 195-205. Asp-197 of IDH2 aligns with an aspartate residue found to be chemically labeled by reaction of pig NAD+-IDH with an analogue of the allosteric modifier ADP (37). This result, in addition to the IDH2 subunit may have a regulatory site as well as catalytic function.

Relationship of NAD+- and NADP+-IDH Isozymes—In addition to the NAD+-IDH, two NADP+-IDH isozymes in S. cerevisiae catalyze the conversion of isocitrate to α-ketoglutarate. Although they function in similar reactions, the three isozymes of isocitrate dehydrogenase are structurally distinct. The mitochondrial NADP+-IDH enzyme is similar to the E. coli enzyme in that it is a homodimer composed of subunits with a molecular weight of ~45,000 and does not appear to be allosterically regulated. The gene encoding mitochondrial NADP+-IDH from S. cerevisiae has been cloned and the nucleotide sequence determined (11); however, no significant similarity (<20%) is observed when the protein sequence is compared with those for E. coli NADP+-IDH or yeast IDH2. The lack of primary structure similarity between the mitochondrial NADP+-IDH and NAD+-IDH isozymes supports the idea that these isozymes function in separate metabolic pathways.

An important implication of the ability of the IDH2 disruption strain to grow on glycerol or lactate is that another enzyme is partially compensating for the lack of NAD+-IDH activity. We presume that this is the NADP+-IDH, with the most likely candidate being the mitochondrial isozyme. Both isocitrate and α-ketoglutarate, however, can cross the mitochondrial membrane, and thus the cytoplasmic isozyme may also contribute α-ketoglutarate to be utilized for citric acid cycle function. The growth phenotype of the ΔIDH2 strain suggests an essential role of NADP+-IDH in the oxidative function of the citric acid cycle and that the NADP+-IDH isozymes can function to provide α-ketoglutarate, although at a reduced rate, to the cycle in the absence of a functional NAD+-IDH isozyme. This hypothesis may be tested by disruption of the NADP+-IDH genes in the strain lacking IDH2 and analysis of the resulting growth phenotypes.

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