Alternatives to the Isomerase-dependent Pathway for the β-Oxidation of Oleic Acid Are Dispensable in *Saccharomyces cerevisiae*

IDENTIFICATION OF YOR180c/DCII ENCODING Peroxisomal Δ3,5-Δ2,4-Dienoyl-CoA Isomerase*

(Received for publication, January 11, 1999, and in revised form, May 12, 1999)

Aner Gurvitz‡‡, Anu M. Mursula‡, Ahmed I. Yagi‡, Andreas Hartig‡, Helmut Ruß‡, Hanspeter Rottensteiner‡‡‡, and J. Kaleervo Hiltunen‡‡

From the ‡‡Institut für Biochemie und Molekulare Zellbiologie der Universität Wien and Ludwig Boltzmann-Forschungsstelle für Biochemie, Vienna Biocenter, Dr Bohrgasse 9, A-1030 Wien, Austria and the ‡Biocenter Oulu, Department of Biochemistry, University of Oulu, FIN-90570 Oulu, Finland

Fatty acids with double bonds at odd-numbered positions such as oleic acid can enter β-oxidation via a pathway relying solely on the auxiliary enzyme Δ3,Δ5-Δ2,4-enoyl-CoA isomerase, termed the isomerase-dependent pathway. Two novel alternative pathways have recently been postulated to exist in mammals, and these additionally depend on Δ3,5-Δ2,4-dienoyl-CoA isomerase (di-isomerase-dependent) or on Δ3,5-Δ2,4-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase (reductase-dependent). We report the identification of the *Saccharomyces cerevisiae* oleic acid-inducible *DCII* (YOR180c) gene encoding peroxisomal di-isomerase. Enzyme assays conducted on soluble extracts derived from yeast cells overproducing Dci1p using 3,5,8,11,14-eicosapentaenoyl-CoA as substrate demonstrated a specific di-isomerase activity of 6 nmol × min⁻¹ per mg of protein. Similarly enriched extracts from *eci1A* cells lacking peroxisomal 3,2-isomerase additionally contained an intrinsic 3,2-isomerase activity that could generate 3,5,8,11,14-eicosapentaenoyl-CoA from 2,5,8,11,14-eicosapentaenoyl-CoA but did not metabolize *trans*-3-hexenoyl-CoA. Amplification of this intrinsic activity replaced Eci1p since it restored growth of the *ecilA* strain on petroselinic acid for which di-isomerase is not required whereas Eci1p is. Heterologous expression in yeast of rat di-isomerase resulted in a peroxisomal protein that was enzymatically active but did not re-establish growth of the *ecilA* mutant on oleic acid. A strain devoid of Dci1p grew on oleic acid to wild-type levels, whereas one lacking both Eci1p and Dci1p grew as poorly as the *ecilA* mutant. Hence, we reasoned that yeast di-isomerase does not additionally represent a physiological 3,2-isomerase and that Dci1p and the postulated alternative pathways in which it is entrained are dispensable for degrading oleic acid.

In the yeast *Saccharomyces cerevisiae*, saturated fatty acids are degraded through a peroxisomal β-oxidation process (1)

---

* This work was supported in part by Fonds zur Förderung der Wissenschaftlichen Forschung, Vienna, Austria, Grants P12061 (to B. Hamilton) and P12118 (to A. H.), by Jubiläumsfonds der Österreichischen Nationalbank, Austria, Grant 6517 (to H. R.), and by the Sigrid Juselius Foundation, Finland, and the Academy of Finland (to J. K. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Present address: Inst. für Biochemie, Limonenstrasse 7, 12203 Berlin, Germany. Tel.: 49-30-838-2937; Fax: 49-30-838-2936; E-mail: hr@zedat.fu-berlin.de

‡‡ This paper is available on line at http://www.jbc.org

---

1 The abbreviations used are: MFE, multifunctional enzyme; 2,4-reductase, 2,4-dienoyl-CoA reductase; 3,2-isomerase, Δ3,5-Δ2,4-dienoyl-CoA isomerase; di-isomerase, Δ3,5-Δ2,4-dienoyl-CoA isomerase; FCBR, polymerase chain reaction; kb, kilobase; GFP, green fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; ORE, oleate response element; PTS, peroxisomal targeting signal.

24514

This paper is available on line at http://www.jbc.org
utilization of fatty acids were prepared as described (6, 7) and contained

constructed by inserting the

pairs YOR180C-A1/K2 and K3/YOR180C-A4 (17). Additional PCR ver-

mutant. pairs YOR180C-A1/A2 or -A3/A4 which yielded the expected product

BJ1991.

with NaOH) to

or 0.2% (w/v) oleic acid and 0.02% (w/v) Tween 80 (adjusted to pH 7.0

listed in Table II. The plasmid pSK::

in vivo

and Eci1p, and we discuss the

mutants devoid of the three auxiliary enzymes Dci1p, Sps19p,

Strains and Gene Disruptions—Escherichia coli strain DH10B was used for all plasmid amplifications and isolations. The S. cerevisiae

strains constructed here were derived from BJ1991 (15) and are listed in Table I. Construction of strains BJ1991pes6A and BJ1991posx1A had been described elsewhere (16). Construction of DCI1-disrupted strains was performed according to published methods (17). The amplification product (dci1::kanMX4) generated by a polymerase chain reaction (PCR) performed on pFA6a-kanMX4 template DNA (17) with the 58- and 59-mer oligonucleotides YOR180C-S1 and YOR180C-S2 was used to transform strain BJ1991. The deletion of DCI1 in geneticin-resistant transformants was verified by PCR using the oligonucleotide pairs YOR180C-A1/K2 and K3/YOR180C-A4 (17). Additional PCR ver-

ification of the DCI1 deletion was performed using the oligonucleotide pairs YOR180C-A1/2A or -A3/A4 which yielded the expected product with wild-type genomic DNA as template but not with that of the mutant.

The BJ1991dci1dci1 strain was generated by transforming the BJ1991dci1/1A delatent with a Spel-SpoI fragment from pAG916 contain-

ing an eccl::kanMX4::URA3 deletion. The eccl deletion was verified in URA3 transformants that failed to grow on oleic acid by PCR using the YLR284C-A1/A4 oligonucleotides (7) which gave rise to a single band representing the deleted gene. The BJ1991eccl/1A::kanMX4::URA3 double mutant was generated by transforming a BJ1991eccl/1A (7) strain with an Spel-SpoI fragment from pAG109 (6) containing the

sps19::LEU2 deletion. Alteration of SPS19 as a result of incorporating the

sps19::LEU2 disruption fragment was verified by Southern analysis of HindIII-digested genomic DNA using a 1.4-kb Spal-XbaI

SPS18/19 fragment from pAG454 (6).

Media and Growth Conditions—RNA was isolated from logarithmic cultures grown for 16 h in medium containing 1% (w/v) yeast extract, 2% (w/v) bactopeptone, and either 2% (w/v) glucose, 2% (w/v) ethanol, or 0.2% (w/v) oleic acid and 0.02% (w/v) Tween 80 (adjusted to pH 7.0 with NaOH) to Aeromax = 1.0. Plates or liquid media used to assess utilization of fatty acids were prepared as described (6, 7) and contained the sole carbon source 0.125% (w/v) of either palmitic acid, arach-

donic acid (cis-5,8,11,14-eicosatetraenoic acid (Sigma)) used to generate the latter substrate from arachidonoyl-CoA (14). The assay system for 3,2-isomerase activity in Dci1 was performed in the absence of exogenous acyl-CoA oxidase using excess purified recombinant rat di-

isomerase and arachidonoyl-CoA as substrate. In this assay system, which monitored metabolism of 2,5,8,11,14-eicosapentenoic-CoA via 3,5,8,11,14-eicosapentenoic-CoA to 2,4,8,11,14-eicosapentenoic-CoA, the rate-limiting step was the Delta-9 isomerization. 2,5,8,11,14-Eicosapentenoic-CoA was generated from arachidonoyl-CoA by endogenous acyl-CoA oxidase (PoxIp) in the lysates of yeast cells grown on oleic acid and was not rate-limiting. To 30 μM arachidonoyl-CoA was sequentially added 2 μg of purified recombinant rat di-isomerase, 10 μg of soluble protein extract from BJ1991eccl/1A harboring the plasmid vector, followed by 10 μg of protein from isogenic cells overexpressing Dci1p. The assay for 3-oenoyl-CoA hydratase 1 was conducted according to published methods (22) using trans-2-hexenoyl-CoA as substrate. The as-

TABLE I

S. cerevisiae strains used

| Strain | Description | Source or Ref. |
|--------|-------------|----------------|
| yAG933 | Contains pSK::DCI1 overexpressing Dci1p This study |
| yAG934 | Contains pADH2-DCI1::GFP This study |
| yAG936 | Contains pADH2-DCI1::GFP This study |
| yAG954 | Contains the Yeplac112 plasmid vector This study |
| yAG976 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |
| yAG978 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |
| yAG980 | Contains the Yeplac112 plasmid vector This study |
| yAG982 | Contains the Yeplac181 plasmid vector This study |
| yAG984 | Contains the YEplac181 plasmid vector This study |
| yAG986 | Contains the YEplac181 plasmid vector This study |
| yAG992 | Contains the pYE352-CTA1 plasmid vector This study |
| yAG993 | Contains the Yeplac112 plasmid vector This study |
| yAG994 | Contains the pAG916; D:: KanMX4::URA3 This study |
| yAG995 | Contains the Yeplac181 plasmid vector This study |
| yAG997 | Contains the pKA-EC1H1 overexpressing rat di-isomerase This study |
| yAG999 | Contains the pYE352-CTA1 plasmid vector This study |
| yAG998 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |
| yAG999 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |
| yAG998 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |
| yAG998 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |
| yAG998 | Contains the Yeplac112::ECI1 overexpressing Eci1p This study |

a The numbers in superscript following designation of the strains refer to their parental genotypes, e.g. BJ1991dci1/1A was derived from 1) BJ1991.
say for peroxisomal MFE type II (combined 2-enoyl-CoA hydratase 2 and β-specific 3-hydroxyacyl-CoA dehydrogenase activities) was performed with trans-2-decenoyl-CoA as substrate (23).

### RESULTS

**YOR180c Encodes an Oleic Acid-inducible Peroxisomal Protein**—Current understanding of mammalian β-oxidation postulates that oleic acid can be metabolized via alternative pathways (solid arrows; Fig. 1C). Yeast cells have been shown previously to require 3,2-isomerase (Eci1p) for breaking down oleic acid (7, 8); however, it was not known whether they also possessed a di-isomerase that could enable them to degrade this fatty acid via the reductase-dependent or the di-isomerase-dependent routes. A previous search of the *S. cerevisiae* Genome Data Base as bases for open reading frames with promoters containing an oleate response element (ORE) (31, 32) that could encode peroxisomal targeted proteins entrained in β-oxidation revealed two novel homologous (46% identity) genes *YLR284c (ECI1)* and *YOR180c* (7). In line with other genes encoding β-oxidation enzymes up-regulated in cells grown in oleic acid medium

### Table II

| Table II | Plasmids and oligonucleotides used |
|----------|-----------------------------------|
| **Plasmid** | **Description** | **Source or Ref.** |
| pFA6a-kanMX | Template for kanMX4-based PCR disruptions | 17 |
| pAG916 | eci1Δ::kanMX4::URA3 in pGEM-T | This study |
| pAG129 | sps1Δ::LEU2 disruption plasmid | 6 |
| pSK:YOR180c | 1.6-kb DCI1 (H039/4) in pSKX01a and Pef1 | A. Fiehinger* |
| pAG774 | 1.6-kb DCI1 (YOR180C A1/A4) in Yeplac181::Sma1 | This study |
| Yeplac181/112 | LEU2::TRP1-containing multicopy vectors | 18 |
| pADH2-GFP-DCI1 | GFP::CIpl fusion linked to the ADH2 promoter | This study |
| pADH2-GFP-ECI1 | GFP::Eci1p fusion linked to the ADH2 promoter | 7 |
| Yeplac204 | TRP1-containing integrative vector | 18 |
| pGEM:eci1Δ | Amplified eci1Δ::kanMX4 (YOR180C A1/A4) | This study |
| pJJ244 | HindIII fragment containing URA3 for pAG916 | 19 |
| pKA ECH1 | Rat ECH1 (ECl1/F-R) in pYE352-CTA1 | This study |
| pYE352-CTA1 | Vector for expressing rat ECH1 from the CTA1 promoter | 20 |
| pSK:YLR284c | 1.5-kb XbaI-Pef1 fragment containing ECI1 | 7 |
| pAG113 | 189-base pair Clal-SphI fragment containing SPS19 | 6 |
| pAD17 | 2.4-kb BglII-BglII fragment containing POX1 | 2 |
| pJA301 | 3.5-kb BanHI-EcoRI fragment containing ACT1 | 29 |
| pAG766 | 1.5-kb fragment containing ECI1 in Yeplac181::Sma1 | 7 |
| pAG769 | 1.5-kb fragment containing ECI1 in Yeplac112::Sma1 | 7 |
| pAG788 | 1.6-kb DCI1 (YOR180C A1/A4) in Yeplac112::Sma1 | 18 |

### Table I

| Table I | Plasmids or oligonucleotides used |
|----------|----------------------------------|
| **Oligonucleotide** | **Description** |
| YOR180C-S1 | 5'-CTGGTGTTAAGCTTATGAGTTATCTCTTAACTATAGACGCA-3' | This study |
| YOR180C-S2 | 5'-CTGTAAGCTTCGAGCAG-3' | This study |
| YOR180C-A1 | 5'-ATGCAATGGTACCCCTCC-3' | This study |
| YOR180C-A2 | 5'-ATGCAATGGTACCCCTCC-3' | This study |
| YOR180C-A3 | 5'-GGGCAAGGCGGCAGCCCATTTCC-3' | This study |
| YOR180C-A4 | 5'-GCCCAGGCCGCTCTTGTCTG-3' | This study |
| H039S (YOR180C 5'-end) | 5'-GCTGGTAAAGCTTATGAGTTATCTCTTAACTATAGACGCA-3' | This study |
| H039A (YOR180C 3'-end) | 5'-CTGTAAGCTTCGAGCAG-3' | This study |
| NotI-1800-CF | 5'-ATGCAATGGTACCCCTCC-3' | This study |
| Salt-1800-R | 5'-ATGCAATGGTACCCCTCC-3' | This study |
| YOR180C-ORE1 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| YOR180C-ORE2 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| ECI1-F | 5'-GGCCTCTGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| ECI1-R | 5'-GGCCTCTGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| YLR284C-ORE1 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| YLR284C-ORE2 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| SPS19ORE1 | 5'-TCAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| SPS19ORE2 | 5'-TCAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| FOX1ORE1 | 5'-ATGCAATGGTACCCCTCC-3' | This study |
| FOX1ORE2 | 5'-ATGCAATGGTACCCCTCC-3' | This study |
| CTA1ORE1 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| CTA1ORE2 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| CTA1mutORE1 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |
| CTA1mutORE2 | 5'-TGAAGCTTACACTTCGAGGACGCAATGACGCAATGACGCAATGAC-3' | This study |

### References

* Reserved in the Saccharomyces Genome Data Base as an enoyl-CoA hydratase homologue; I. V. Karpichev, J. Lopez, and G. M. Small, manuscript in preparation.
Appropriately, levels of $DCI1$ transcript were very low in a similarly propagated pip2Δaof1Δ mutant (34) devoid of the DNA-binding proteins mediating the transcriptional response to oleic acid. The induction of $DCI1$ was consistent with the presence in its promoter of an ORE matching the consensus and 35; Fig. 2A). During the course of this work, $YOR180c/DCI1$ was found to be induced (Fig. 2A).

appropriately not involved in β-oxidation of fatty acids. To determine whether $YOR180c/DCI1$ represented the gene for di-isomerase, this fluorescence was diffuse (Fig. 2C). Control DAPI staining for DNA demonstrated a similar mitochondrial (as well as nuclear) fluorescence pattern in both strains. This indicated that Dci1p was compartmentalized in peroxisomes. Since in addition to being oleic acid-inducible as well as peroxisomal, Dci1p is also a member of the hydratase/isomerase family of proteins (42) with the potential to utilize acyl-CoA substrates, it possessed the characteristics of yeast proteins involved in β-oxidation of fatty acids.

Overexpression of Dci1p in an eci1Δ Strain Restores Growth on Oleic Acid—This study is concerned with the identification of yeast di-isomerase. A search of the data bases revealed three yeast genes encoding proteins with similarity to rat di-isomerase, these being $ECH1$, $YOR180c/DCI1$, and $YDR306c$. Eci1p has been shown previously not to contain di-isomerase activity (7), whereas $YDR306c$, which does not encode a protein with an obvious PTS and did not show up in our ORE search, is probably not involved in β-oxidation of fatty acids. To determine whether $YOR180c/DCI1$ represented the gene for di-isomerase, it was overexpressed in an eci1Δ strain that was impaired for growth on oleic acid. We reasoned that since the two 3,2-isomerase activities entrained in the reductase-dependent pathway (diagonal arrows; Fig. 1C) may not necessarily be encoded by $ECH1$, amplification of a di-isomerase could relieve the requirement for Eci1p during growth on oleic acid.

Mutants overexpressing Dci1p were compared with those enriched with Eci1p or harboring the plasmid vector for utilization of oleic acid using a zone-clearing assay (Fig. 3). In this and subsequent assays, fatty acid plates were used that additionally contained Tween 80 which acted to form an emulsion but also as a poor carbon source. Thus, cells could form small colonies on these plates but zones of clearing (i.e. opaque halos

Fig. 1. Pathways for degrading fatty acids. A, the β-oxidation spiral for breaking down saturated fatty acids. The yeast enzymes Pox1p (acyl-CoA oxidase), Fox2p (2-enoyl-CoA hydratase 2 and b-specific 3-hydroxyacyl-CoA dehydrogenase), and Pot1p/Fox3p (3-ketoacyl-CoA thiolase) are noted. Enzymes are indicated to the left of the dashed arrows, and metabolites are noted above them in italics. B, the position of the auxiliary enzymes Sps19p (2,4-di-enoyl-CoA reductase) and Eci1p (Δ3,Δ5-enoyl-CoA isomerase) in the degradation pathway of unsaturated fatty acids with double bonds at even-numbered positions. Double arrowhead represents the two steps of Fox2p. C, the three possible pathways for degrading fatty acids with double bonds at odd-numbered positions. The isomerase-dependent pathway (dashed arrows) has been shown previously to be required for growth of yeast on oleic acid medium. The postulated mammalian reductase- and di-isomerase-dependent pathways are indicated by solid arrows.
in the medium), such as those generated by the mutant expressing Dci1p or Eci1p (Fig. 3), indicated utilization of the fatty acid substrate. Hence, this demonstrated that Dci1p could have a direct role in \(\beta\)-oxidation of fatty acids. Cells harboring the respective plasmid vector did not form a clearing zone in the medium and were, therefore, considered impaired for utilization of oleic acid. Growth of the ec1Δ strain on oleic acid could have been restored due to at least two possibilities as follows: (i) overexpression of Dci1p channeled the carbon flow from oleic acid via the di-isomerase and/or reductase-dependent pathways (Fig. 1C), or (ii) Dci1p represented a second 3,2-isomerase.

To determine the mechanism of the relief of the requirement for Eci1p, an eci1Δsps19Δ double disruptant was generated that was additionally devoid of 2,4-reductase activity (Fig. 1C). In this mutant, the reductase-dependent pathway would be blocked. In agreement with current knowledge of the breakdown of fatty acids with double bonds at even-numbered positions which requires Sps19p (Fig. 1B), amplification of Dci1p did not re-establish growth of the mutant on petroselinic acid but did on oleic acid (performed with strains yAG952 and yAG953). Assuming that Sps19p represented the 2,4-reductase and that was additionally devoid of 2,4-reductase activity (Fig. 1C).

Dci1p Is Dispensable for Yeast Growth on Oleic Acid—To elucidate the extent to which Dci1p was involved in the metabolism of fatty acids, a DCI1-deleted strain was constructed and examined for growth on fatty acid media using a zone-clearing assay. Wild-type and dci1Δ strains were applied as serially diluted culture spots onto plates containing either palmitic, oleic, or arachidonic acid (Fig. 4). In this zone-clearing assay, utilization of the fatty acids was represented by thin opaque rings around the colonies. These plate assays showed that the dci1Δ strain was capable of forming a clear zone in all solid media examined (including in cis-12-octadecenoic acid; cis-C18:1(12), not shown). A pox1Δ strain lacking acyl-CoA oxidase (Fig. 1A) which is defective for \(\beta\)-oxidation (2) was added to the plates to demonstrate lack of zone formation on any of the fatty acids tested. A similarly applied eci1Δ strain with impaired growth on unsaturated fatty acids (Fig. 1, B and C) but not on saturated ones (7, 8) formed wild-type clear zones only on palmitic acid. Although some clearing was also produced by the eci1Δ strain on oleic acid, albeit below that by the dci1Δ strain, it failed to utilize arachidonic acid. The sps19Δ disruptant (yAG141) lacking 2,4-reductase (Fig. 1B) and impaired for growth on fatty acids with double bonds at even-numbered positions was unable to utilize arachidonic acid but was otherwise unaffected on oleic acid and palmitic acid.

Vital counts following growth in liquid oleic acid medium were used to corroborate the observations made with the plates (Table III). In a representative experiment, at the time point (75 h) when the pox1Δ and eci1Δ strains were clearly affected since their respective number of surviving colonies had reached only 9 and 18% that of wild type, those of the dci1Δ and sps19Δ mutants were as high as 110 and 116%, respectively. This indicated that, like with the sps19Δ disruption, deletion at DCI1 did not affect the number of surviving cells following prolonged propagation in liquid medium containing oleic acid and supported the observations made using solid media.

Dci1p-enriched Cell Extracts Contain Di-isomerase and Isomerase Activities—To determine the enzymatic properties of Dci1p, it was overexpressed in the corresponding dci1Δ strain from a multi-copy plasmid. Soluble protein extracts derived
from homogenized oleic acid-induced cells were used in assays for possible hydratase/isomerase enzyme activities, including those of 2-enoyl-CoA hydratase 1 (with trans-2-hexenoyl-CoA as substrate) and di-isomerase (using 3,5-hexadienoyl-CoA or 3,5,8,11,14-eicosapentenoyl-CoA), as well as peroxisomal MFE type II. By using 3,5,8,11,14-eicosapentenoyl-CoA as substrate, a specific di-isomerase activity was measured in the Dci1p-enriched extracts (Fig. 5, arrow 2) as an increase in A_{300 nm}. This activity was comparable to that contained in pure recombinant rat di-isomerase (Fig. 5, arrow 3) and was equivalent to 6 nmol × min⁻¹ per mg of protein. Extracts from a mutant harboring the plasmid vector did not contain detectable levels of this activity (Fig. 5, arrow 1). It has been shown previously that the increase in A_{300 nm} under these experimental conditions is due to the transfer of Δ^2-Δ^4-conjugated double bonds of dienoyl-CoAs to the Δ^2-Δ^4 positions (14). Di-isomerase activity could also be measured in the Dci1p-enriched extracts using 3,5-hexadienoyl-CoA as substrate (not shown).

Assays for 2-enoyl-CoA hydratase 1 or peroxisomal MFE type II activities performed on the control and Dci1p-enriched extracts did not demonstrate any differences. The similarity between Dci1p and Eci1p also prompted the determination of whether Dci1p additionally possessed a detectable level of 3,2-isomerase activity. By using trans-3-hexenoyl-CoA as substrate, the activity in soluble protein extracts from homogenized eci1Δ cells overexpressing Dci1p was below the detection limit of the assay; however, these extracts could generate 3,5,8,11,14-eicosapentenoyl-CoA from 2,5,8,11,14-eicosapentenoyl-CoA and therefore contained an intrinsic Δ^3-Δ^5-enoyl-CoA isomerase activity (performed according to the method under "Experimental Procedures") using strains yAG922 and yAG958, not shown.

Overexpressed Dci1p Functionally Replaces Eci1p Due to Amplification of a 3,2-Isomerase Activity—Detection of the intrinsic 3,2-isomerase activity in Dci1p raised the question of whether re-establishment of the eci1Δ strain’s growth on oleic acid by overexpressing Dci1p (Fig. 3) could have been mediated not only by engagement of the alternative routes but also by replacing the missing 3,2-isomerase activity (Fig. 1C). Therefore, the effect of overexpressing Dci1p in the eci1Δ strain was examined also on petroselinic acid for which the alternative pathways are not thought to be engaged, whereas Eci1p is (Fig. 1B). Since enrichment with Dci1p restored the ability of the eci1Δ strain to utilize petroselinic acid in a similar manner to Eci1p (Fig. 6), this underscores the enzyme assay result that Dci1p possessed some 3,2-isomerase activity. Hence, in the previous growth assay on oleic acid (Fig. 3), in addition to possibly activating the two alternative routes, overexpressed Dci1p could have also acted to restore the growth of the mutant by supplying the missing 3,2-isomerase activity.

Dci1p Is Not a Redundant Eci1p Isoenzyme—To determine whether Dci1p represented a physiological 3,2-isomerase, a BJ1991dcilΔeci1Δ double mutant was generated and compared with the BJ1991eci1Δ single mutant for utilization of
oleic acid, on which the latter strain grows to some extent (Fig. 4). If deletion of both \( EC\) and \( DC\) were to block completely degradation of unsaturated fatty acids, then the double mutant would be expected to appear as a \( p\) strain, where no zone was detected even at the highest density of cells spotted on solid oleic acid medium (Fig. 4). However, close scrutiny of similarly dense \( dc\) double mutant and \( ec\) single mutant cells demonstrated that both utilized this fatty acid to some extent since a faint ring could be seen (Fig. 7). This minimal zone was probably due to their ability to undergo three rounds of \( \beta\)-oxidation prior to encountering the \( \Delta^5\) double bond in oleic acid. Growth of the single and double mutants was also examined in liquid oleic acid medium. Following propagation for 48 h, the number of viable colonies of the double mutant was comparable to that of the corresponding single deleter (11 and 13% of the wild type, respectively). This implied that as a single genomic copy, \( DC\) probably did not act as a gene for a redundant \( Ec\) enzyme despite containing an intrinsic 3,2-isomerase activity. However, the finding that \( Dc\) could generate 3,5,8,11,14-eicosapentenoyl-CoA from 2,5,8,11,14-eicosapentenoyl-CoA indicated that it could represent a novel isomerase necessary for generating the 3,5-dienoyl-CoA substrate required for the di-isomerase (upper diagonal arrow; Fig. 1C). In this case, overexpression of a di-isomerase without an intrinsic 3,2-isomerase activity in the \( ec\) mutant (in which \( Dc\) is intact) should re-establish growth on oleic acid due to amplification of the di-isomerase pathway. Rat di-isomerase could be used for this purpose since the purified recombinant protein does not contain 3,2-isomerase activity (14) and since heterologous expression of rat peroxisomal proteins has been shown previously to work in yeast cells, e.g. rat peroxisomal MFE type I can replace Fox2 and \( Ec\) (7, 20).

Rat \( E\) encoding di-isomerase (14) was expressed in the \( e\) mutant. Enzyme assays performed on extracts derived from yeast cells using 3,5,8,11,14-eicosapentenoyl-CoA as substrate verified that the rat di-isomerase is active, and subsequent immuneelectron microscopy demonstrated that the rat protein entered their peroxisomes (not shown). However, in a zone-clearing assay on oleic acid or petroselinic acid media using strains yAG977 to yAGS80 (Table II) expression of rat di-isomerase did not result in the restoration of the \( e\) mutant’s growth. Hence an amplified rat di-isomerase could not act to relieve the requirement for \( Ec\) probably because \( Dc\) did not represent the 3,2-isomerase activities acting in concert with di-isomerase in the alternative pathways (diagonal arrows; Fig. 1C). We reason that although overexpressed \( Dc\) could have acted to re-establish growth of the \( e\) mutant on oleic acid (Fig. 3) through each of the three routes (Fig. 1C), it probably did so solely via the isomerase-dependent pathway as a result of a grossly amplified intrinsic 3,2-isomerase activity (as with petroselinic acid; Fig. 5).

**DISCUSSION**

Here we report the identification of the oleic acid-inducible \( S.\) \( c\) gene encoding peroxisomal di-isomerase. Extracts derived from \( Dc\)-enriched yeast cells contained both di-isomerase and 3,2-isomerase activities, and although overexpression of \( Dc\) in the \( ec\) mutant acted to replace the missing 3,2-isomerase activity on petroselinic acid, we chose to designate the novel gene as a di-isomerase and not as a 3,2-isomerase for the following reasons. First, a previous cell fractionation study revealed that 3,2-enoyl-CoA isomerase activity was not detected in any of the fractions derived from an \( ec\) mutant, including the peak peroxisomal fractions (8). Therefore, if \( Dc\) represented a minor 3,2-isomerase in a partially redundant system with the major 3,2-isomerase \( Ec\), then the \( ec\) mutant must have been able to grow at least slowly on unsaturated fatty acids. This was clearly not the case on arachidonic acid (Fig. 4). Second, in such a system the double \( e\) mutant would have been expected to be more impaired than the single \( e\) mutant for growth on oleic acid, which it wasn’t (Fig. 7).

By using enzyme assays with trans-3-hexenoyl-CoA as substrate, 3,2-isomerase activity in extracts enriched with \( Dc\) was below the detection limit, whereas those similarly enriched with \( Ec\) contained a 3,2-isomerase activity of 60 nmol × min⁻¹ per mg of protein (7). An in vitro assay based on excess purified recombinant rat di-isomerase could demonstrate that \( Dc\) contained a 3,2-isomerase activity. Hence, since 3,2-isomerase activity was too low to be detected through a conventional assay, this provided a third compelling reason not to designate the product of \( YOR180c\) as an \( E\) like 3,2-isomerase. This situation is similar to that of rat di-isomerase which also contains a low level of hydratase 1 activity (14) and is in line with the catalytic properties of several other hydratase/isomerase family members that represent a hydratase 1 and also a 3,2-isomerase, including mammalian peroxisomal MFE type I (22), \( \alpha\)-subunit of bacterial \( \beta\)-oxidation complex (46), and mammalian mitochondrial hydratase 1, in which the latter activity is low (47).

We also report on the dispensability for growth of yeast on oleic acid of the putative mammalian alternative pathways in which di-isomerase is entrained. Deletion of \( DC\) did not affect growth of the corresponding mutant on any of the fatty acids tested. Although this cannot be entirely ruled out, the possibility that a second di-isomerase might exist in \( S.\) \( c\) is low. \( Ec\), the closest homologue of \( Dc\), has been purified previously to homogeneity as a recombinant protein but was found to be without di-isomerase activity (7). YDR036c represents the gene for the third and last unidentified \( S.\) \( c\) member of the hydratase/isomerase protein family is probably

**Fig. 6.** 3,2-Isomerase activity in \( Dc\) restored growth of the \( ec\) mutant on petroselinic acid. Formation of clearing zones by the mutant strain BJ1991lec\( e\) over-expressing \( Dc\) was compared with those overexpressing \( Ec\) or harboring the plasmid vector on petroselinic acid medium. Strains used are listed in the legend to Fig. 3.
also not an oleic acid-inducible, peroxisomal di-isomerase isozyme.

The reductase-dependent pathway in which di-isomerase is implicated is the subject of intensive research (9–13, 43, 44), and the contribution of this pathway to the degradation of fatty acids with cis-double bonds at odd-numbered positions is controversial. One study using intact liver and heart mitochondria reported that cis-4-decenolic acid (cis-C10:1(4)) was completely metabolized by the reductase-dependent pathway (12), whereas more recent work with soluble extracts of rat peroxisomes using 2,5-octadienoyl-CoA as substrate demonstrated that most (80%) of this intermediate was metabolized via the isomerase-dependent pathway (13). Since rat peroxisomes have all the enzymes required for the reductase-dependent pathway (45), it was reasonable to assume that these routes could also occur in yeast peroxisomes.

Generation of yeast cells devoid of di-isomerase activity implied that it was possible to determine the in vivo requirement of these alternative pathways for peroxisomal degradation of fatty acids. The ability of the dci1Δ and sps19Δ strains with defective reductase pathway enzyme activities to grow on unsaturated fatty acids with cis-double bonds at odd-numbered positions was examined on solid as well as in liquid oleic acid media. Under the conditions tested, both the dci1Δ and sps19Δ strains grew much faster than the eci1Δ and pox1Δ mutants. This was in agreement with data generated during the course of this work in which a yor180c disruptant was compared with a defective pex mutant for growth on oleic acid (38). Although in our BJ1991 strain background the dci1Δ and sps19Δ mutations did not impair growth on oleic acid, those generated in a BY4733 strain caused the resulting disruptants to be partially defective (38). It would have been interesting to see whether more work on soluble extracts of rat peroxisomes using 2,5-octadienoyl-CoA as substrate demonstrated that most (80%) of this intermediate was metabolized via the isomerase-dependent pathway (13). Since rat peroxisomes have all the enzymes required for the reductase-dependent pathway (45), it was reasonable to assume that these routes could also occur in yeast peroxisomes.

We also showed that although a combination of Dci1p and rat di-isomerase could have acted as a di-isomerase-dependent route for breaking down oleic acid, overexpression of the rat protein did not restore growth of the eci1Δ mutant. Taken together, the data presented here serve to underscore our conclusion that yeast di-isomerase is dispensable rather than redundant and that the reductase- and di-isomerase-dependent pathways are not physiologically relevant for growth of yeast on unsaturated fatty acids such as oleic acid. Although this conclusion could be cautiously extrapolated to mammalian peroxisomal β-oxidation, the role of these alternative pathways in mammalian mitochondrial β-oxidation could be more important.

Acknowledgements—We thank Hannelore Wrba, Marika Kamps, and Marion Repitz for excellent technical assistance and Andreas Firzinger for conducting the data base search and for plasmid pSK:YOR180c.

REFERENCES
1. Kunau, W.-H., Bühne, S., Moreno de la Garza, M., Kionka, C., Matebloowski, M., Schultz-Borchard, U., and Thieringer, R. (1988) Biochem. Soc. Trans. 16, 418–420
2. Dmoschowska, A., Dignard, D., Maleszka, R., and Thomas, D. Y. (1990) Gene (Amst.) 88, 247–252
3. Hiltunen, J. K., Wenzel, B., Beyer, A., Erdmann, R., Fossa, A., and Kunau, W.-H. (1992) J. Biol. Chem. 267, 6646–6653
4. Igual, J. C., Matallan, E., Gonzalez-Bosch, C., Franco, L., and Perez-Ortín, J. E. (1991) Yeast 7, 379–389
5. Einerhand, A. W. C., Voorn-Brouwer, T. M., Erdmann, R., Kunau, W.-H., and Tabak, H. F. (1991) Eur. J. Biochem. 206, 113–122
6. Gurvitz, A., Rottensteiner, H., Kilpeläinen, S. H., Hartig, A., Hiltunen, J. K., Binder, M., Dawes, I. W., and Hamilton, B. (1997) J. Biol. Chem. 272, 22140–22147
7. Gurvitz, A., Mursula, A. M., Firzinger, A., Hamilton, B., Kilpeläinen, S. H., Hartig, A., Rius, H., Hiltunen, J. K., and Rottensteiner, H. (1998) J. Biol. Chem. 273, 31366–31374
8. Geisbrecht, B. V., Zhu, D., Schulz, K., Nau, K., Morrell, J. C., Geraghty, M., Schulz, H., Erdmann, R., and Gould, S. J. (1998) J. Biol. Chem. 273, 33184–33191
9. Tserng, K.-Y., and Jin, S.-J. (1991) J. Biol. Chem. 266, 11164–11169
10. Smeland, T. E., Nada, M., Cuebas, D., and Schulz, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6673–6677
11. Luthria, D. L., Baykousheva, S. P., and Sprecher, H. (1995) J. Biol. Chem. 270, 13771–13776
12. Tserng, K.-Y., Jin, S.-J., and Chen, L.-S. (1996) Biochem. J. 313, 581–588
13. Shoukry, K., and Schulz, H. (1998) J. Biol. Chem. 273, 6892–6899
14. Filippula, S. A., Yagi, A., Kilpeläinen, S. H., Novikov, D., FitzPatrick, D. R., Vilhunen, M., Valle, D., and Hiltunen, J. K. (1998) J. Biol. Chem. 273, 49–55
15. Jones, E. W. (1977) Genetics 85, 23–33
16. Gurvitz, A., Rottensteiner, H., Hiltunen, J. K., Binder, M., Dawes, I. W., Rius, H., and Hamilton, B. (1997) Mol. Microbiol. 26, 675–685
17. Wach, A., Brachat, A., Pohlmann, R., and Philippens, P. (1994) Yeast 10, 1739–1803
18. Gietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534
19. Jones, J. S., and Prakash, L. (1990) Yeast 6, 363–366
20. Filippula, S. A., Sormunen, R. T., Hartig, A., Kunau, W.-H., and Hiltunen, J. K. (1995) J. Biol. Chem. 270, 27453–27457
21. Hiltunen, J. K., Osmundsen, H., and Bremer, J. (1993) Biochim. Biophys. Acta 752, 223–232
22. Palosaari, P. M., and Hiltunen, J. K. (1990) J. Biol. Chem. 265, 2446–2449
23. Moreno de la Garza, M., Schulz-Borchardt, U., Crabb, J. W., and Kunau, W.-H. (1985) Eur. J. Biochem. 148, 285–291
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.