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Preliminary Characterization of Yor180Cp: Identification of a Novel Peroxisomal Protein of *Saccharomyces cerevisiae* Involved in Fatty Acid Metabolism

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Here we report the preliminary characterization of Yor180Cp, a novel peroxisomal protein involved in fatty acid metabolism in the yeast *Saccharomyces cerevisiae*. A computer-based screen identified Yor180Cp as a putative peroxisomal protein, and Yor180Cp targeted GFP to peroxisomes in a *PEX8*-dependent manner. Yor180Cp was also detected by mass spectrometric analysis of an HPLC-separated extract of yeast peroxisomal matrix proteins. *YOR180C* is upregulated during growth on oleic acid, and deletion of *YOR180C* from the yeast genome resulted in a mild but significant growth defect on oleic acid, indicating a role for Yor180Cp in fatty acid metabolism. In addition, we observed that *yor180c*Δ cells fail to efficiently import the enzyme Δ^3,Δ^2-enoyl-CoA isomerase (Eci1p) to peroxisomes. This result suggested that Yor180Cp might associate with Eci1p *in vivo*, and a Yor180Cp–Eci1p interaction was detected using the yeast two-hybrid system. Potential roles for Yor180Cp in peroxisomal fatty acid metabolism are discussed.

The use of fatty acids as a source of cellular energy is highly conserved throughout evolution and is accomplished principally through the pathway of β-oxidation. In lower eukaryotes such as yeast, β-oxidation is confined to the peroxisome (1,2). Numerous genetic and biochemical studies on the yeast *S. cerevisiae* have identified the enzymes that catalyze the individual reactions of fatty acid β-oxidation (3–5), as well as certain auxiliary enzymes that facilitate the fatty acid oxidation process (6–10). However, not all of the auxiliary enzymes predicted to exist in yeast peroxisomes have been characterized at the molecular level (9), and recent studies indicate that there may be unappreciated enzymatic contributions to peroxisomal fatty acid oxidation (11). We recently implemented a genome-wide screen for novel peroxisomal proteins in yeast (12). Studies on a number of these candidates have resulted in the characterization of a yeast peroxisomal Δ^3,Δ^2-enoyl-CoA isomerase (Eci1p) (9) and a novel peroxisomal thioesterase (Pte1p) (11). In this report we describe our analysis of Yor180Cp, a candidate identified in our screen for novel peroxisomal proteins.

MATERIALS AND METHODS

All PCR reactions were performed with a low error-rate mixture of polymerases (Expand, Boehringer-Mannheim). The wild type (BY4733) (13) and *pex8*Δ (BY4733, *pex8*Δ::*HIS3*) (12) strains of *S. cerevisiae* have been described. The *YOR180C* open reading frame (ORF) was deleted from the BY4733 genome by PCR-mediated, one-step gene disruption (13) to create the strain *yor180c*Δ (BY4733, *yor180c*Δ::*HIS3*). Specifically, the oligonucleotides YOR180Cko.5 and YOR180Cko.3 were used to amplify the *HIS3* gene using the non-replicating plasmid pRS303 (14) as a template. These oligonucleotides have the sequence 5′-GAGTAGGATTTTCTGGTGTT-AAGTCTAGTGAGTTATCTTCTAACTAGATTGTACTGAGAGTG-CAC-3′ and 5′-TAGTAGATTTTTTATGTTAAAATCCTATCTCTAA-ATGCTATATTACTGTGCGGTATTTCACACCG-3′, respectively. The product of this PCR reaction was used to transform competent BY4733 yeast cells to histidine prototrophy. Precise deletion of the *YOR180C* gene was confirmed by PCR using the *YOR180C*-specific oligonucleotide 5′-CCCGAGCTCAGTTGTGAAGCACTTTAGTTAC-3′ and the *HIS3*-specific oligonucleotide 5′-CGGTGTGAAATACC-GCACAG-3′ as primers to amplify a diagnostic fragment from histidine-prototrophic colonies. Analysis of the growth properties of *yor180c*Δ was performed essentially as described (9).

The oligonucleotides 5′-AAAGTCGACAATGAGCAGTCGTGTG-TGCTAC-3′ and 5′-TTTGCGGCCGCTTATTATAACTTGTGGCGC-CTG-3′ were used to amplify the *YOR180C* ORF from BY4733 genomic DNA. The product of this PCR reaction was digested with *Sal*I and *Not*I and cloned into these sites of the *URA3*-based, oleate-inducible, N-terminal GFP fusion vector, pGFP-X (12), and created

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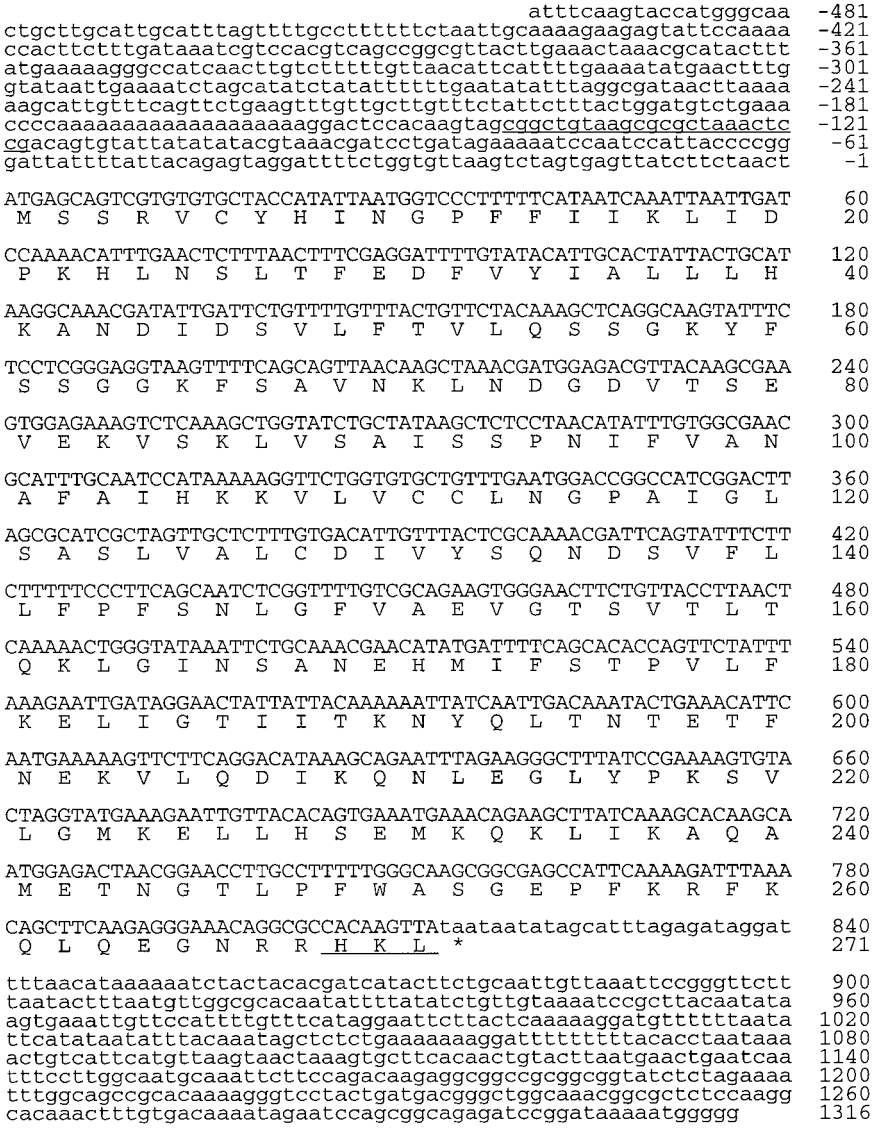

FIG. 1. Nucleotide and predicted protein sequence of the *YOR180C* gene. The *YOR180C* open reading frame is shown with 500 base pairs of flanking sequence at both the 5′- and 3′-ends. A consensus oleate response element (underlined) is found from nucleotides −142 to −119 relative to the first nucleotide of the open reading frame. The 813-base pair open reading frame, which terminates in the atypical PTS-1 His-Lys-Leu-COOH (underlined), is predicted to encode a basic (pI = 8.93) protein with a mass of 30.1 kDa.

pGFP-YOR180C. The absence of mutations in the *YOR180C* ORF of this plasmid was confirmed by automated fluorescent sequencing. Confocal microscopy analysis of yeast cells expressing the GFP-Yor180Cp chimera was performed essentially as described (9). The *YOR180C* open reading frame was excised from pGFP-YOR180C and transferred to the *Sal*I and *Not*I sites of pJL59-*TRP1* (15,16). Likewise, the *ECI1* open reading frame was excised from the plasmid pBG27 (9) and transferred into the identical sites of pPC86-*LEU2* (15,16). These plasmids encode fusions of the DNA-binding and transactivation domains, respectively, of the yeast transcription factor Gal4p. Construction and two hybrid analysis of reporter yeast strains derived from the parental strain MaV99 (17) expressing the fusion proteins BD-Yor180Cp and AD-Eci1p (and appropriate controls) was carried out as described (16), with the exception that the β-galactosidase reporter activity was assayed spectrophotometrically (18).

Soluble peroxisomal proteins were obtained by low-salt extraction of purified *S. cerevisiae* peroxisomes (strain SKQ2N) and were separated by reverse phase HPLC as described (19). After further separation of each fraction by SDS-polyacrylamide gel electrophoresis, selected coomassie-stained proteins were cut out of the gel and digested with trypsin essentially as described (20). Protein fragments were subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI), and identification of proteins was performed by the comparison of the revealed peptide masses with known peptide databases using the ProFound program (21).

For the fractionation of *S. cerevisiae* cells by sucrose density gradient ultracentrifugation, an overnight culture grown in SD medium was used to inoculate 1.5 L of YNOD medium to 1×10^6 cells/ml, and cells were grown with shaking at 14 h at 30°C (9). 5 g of cells were harvested, washed with H_2O, and incubated for 10 min in a solution of 100 mM Tris-HCl (pH 9.4) and 10 mM dithiothreitol at 30°C. Cells

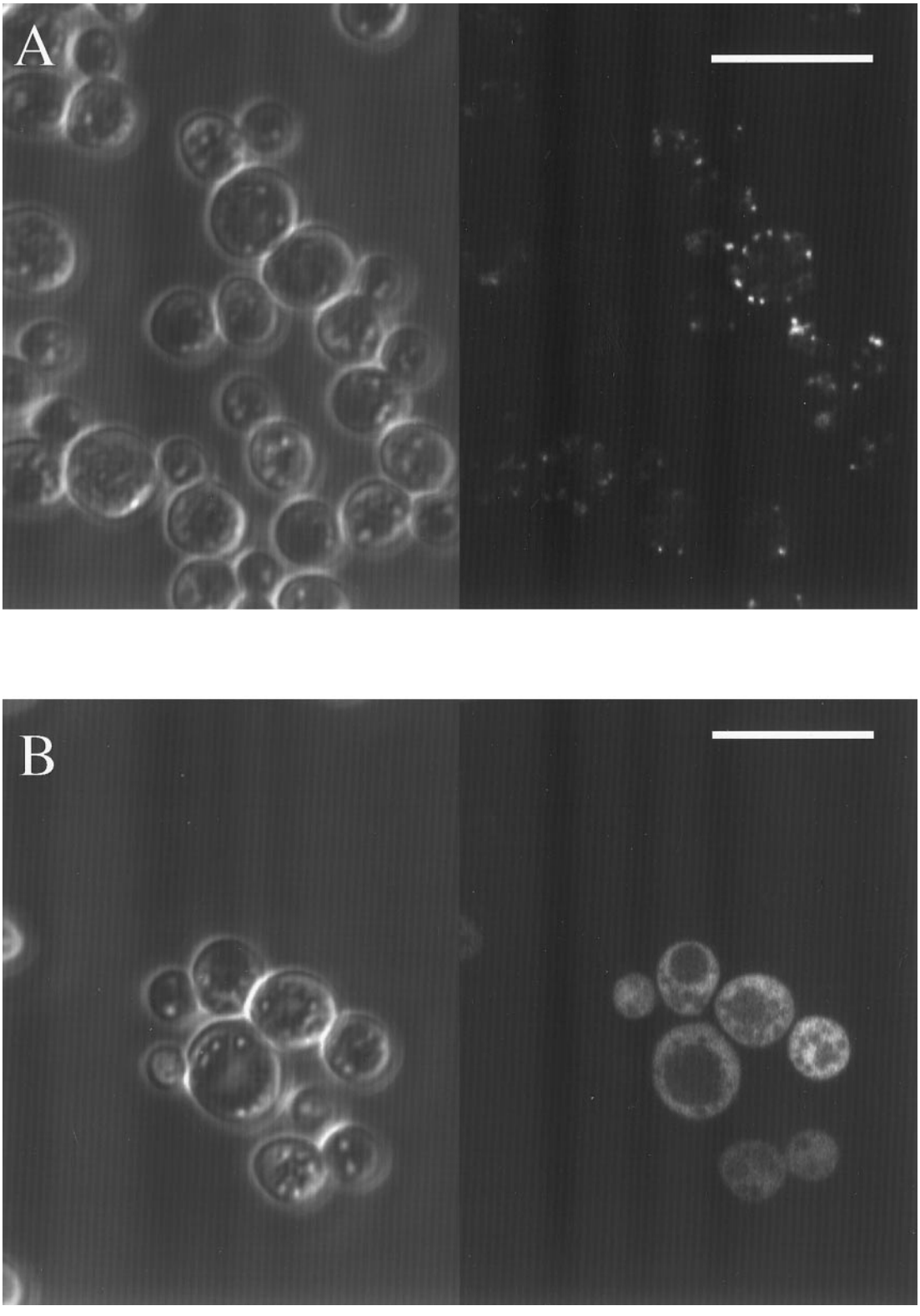

FIG. 2. Yor180Cp targets GFP to peroxisomes in a *PEX8*-dependent manner. A GFP-tagged form of Yor180Cp was expressed in wild-type yeast cells (A) and a *pex8*Δ mutant (B). The distribution of GFP-Yor180Cp was assessed by confocal phase-contrast (left panels) and fluorescence (right panels) microscopy. Bar, 5.0 μm.

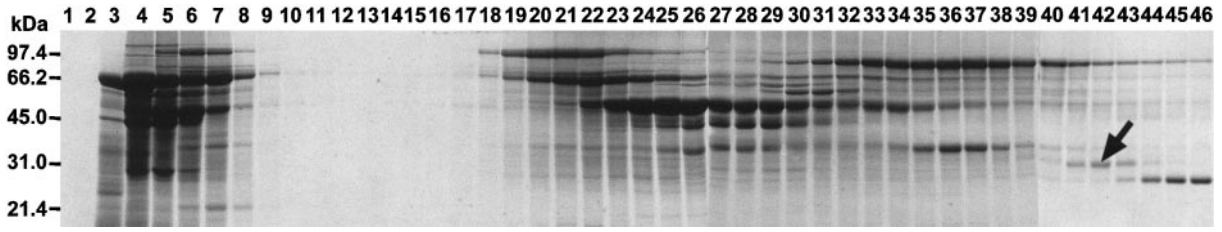

FIG. 3. Preparative chromatographic separation of putative peroxisomal matrix proteins: identification of peroxisomal Yor180Cp by mass spectrometry. A low-salt extract of purified yeast peroxisomes was separated by reverse-phase HPLC. Polypeptides of selected fractions were separated by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining. Following tryptic digestion of selected bands, proteins were identified by MALDI mass spectrometry. The position of the *YOR180C* gene product is indicated by an arrowhead. The amount per lane corresponds to 5% of the total fraction. Molecular weight standards are indicated to the left.

were washed with 1.2 M sorbitol, and cell wall digestion was performed for 1–2 h with 1000 units of lyticase (Sigma) per g of cells in 20 mM potassium P_i (pH 7.4) and 1.2 M sorbitol. Cells were washed twice with 1.2 M sorbitol and resuspended in Dounce buffer (9) that was supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1.25 μg/ml each antipain, chymostatin, pepstatin, leupeptin, and aprotinin, 80 μg/ml benzamidine HCl, and 200 μg/ml sodium fluoride). Homogenization was performed with five passes in a Potter homogenizer (1500 rpm). 8 ml of a post-nuclear supernatant, prepared as described (9), were applied to a 25 ml linear 20–53% (w/w) sucrose gradient in Dounce buffer over a 1 ml 60% (w/w) sucrose cushion. 1 ml fractions were collected from the bottom of the gradient following centrifugation at 48,000 rpm in a Sorvall SV288 rotor for 90 min at 4°C.

Enzyme activity assays for Δ^3,Δ^2-enoyl-CoA isomerase using 3-*cis*-decenoyl-CoA as the substrate were performed as originally described by Binstock and Schulz (22). Assays for fumarase (23) and catalase (23) have been described. Determination of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside as the substrate was performed as described (18). Total protein concentration was determined using the BCA method (Pierce) with bovine serum albumin as a reference. 1 unit of enzyme activity is the amount catalyzing the conversion of 1 μmol of substrate to product in 1 min under standard assay conditions.

RESULTS

During a search for yeast genes likely to encode novel peroxisomal proteins, we identified *YOR180C* as an uncharacterized, oleate-responsive locus whose protein product was predicted to terminate in an atypical, type-1 peroxisomal targeting signal (PTS1) (24), His-Lys-Leu-COOH (Fig. 1). The presence of the PTS-1-like sequence at its C terminus suggested that Yor180Cp was a novel peroxisomal protein. We initially tested this hypothesis by monitoring the ability of Yor180Cp to target GFP to peroxisomes (Fig. 2). GFP is an excellent marker for these studies as it allows direct visualization of protein distribution by fluorescence microscopy and lacks any subcellular targeting information of its own. Following expression of a GFP-Yor180Cp chimera in wild type yeast cells, we found that the pattern of fluorescence was localized to discrete, punctate structures with the characteristic abundance and morphology of peroxisomes (19). To test whether these structures were in fact peroxisomes, we expressed GFP-Yor180Cp in a *pex8*Δ strain, which fails to import peroxisomal matrix proteins (12). We observed a diffuse, cytosolic pattern of GFP-Yor180Cp fluorescence in this mutant. Taken together, these results suggested that *YOR180C* encoded a peroxisomal protein.

The gene product of *YOR180C* was also identified in purified yeast peroxisomes (Fig. 3). A low-salt extract of purified peroxisomes was separated by reverse phase HPLC followed by SDS-polyacrylamide gel electrophoresis. The protein band indicated by an arrowhead in Fig. 3 was excised, digested with trypsin, and the resulting peptides were subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI). Of the 23 monoisotopic masses identified, seven could be assigned to Yor180Cp (745.29, 844.83, 1000.50, 106.45, 1601.61, 1793.80, and 2099.10), and these peptides cover about 32% of the predicted protein. These results are consistent with the GFP targeting experiments shown in Fig. 2, and demonstrate that Yor180Cp is a component of yeast peroxisomes.

The observations that *YOR180C* encodes an novel peroxisomal protein whose abundance increases during growth on fatty acids (12) suggested that Yor180Cp might serve a role in yeast fatty acid metabolism. In order to test this idea directly, we removed *YOR180C* from the *S. cerevisiae* genome and determined the oleate growth rates for the *yor180c*Δ strain, the wild type strain, and a control *pex8*Δ strain (*pex* mutants of *S. cerevisiae* do not grow on oleic acid (25)) (Fig. 4). The strain lacking *YOR180C* exhibited a mild but significant growth defect on oleic acid and grew to a final cell density only 75% of the wild type strain.

The phenotypic analysis of *yor180c*Δ cells was consistent with an ancillary role for Yor180Cp in the peroxisomal metabolism of fatty acids. To test possible functions of this protein, we prepared a post-nuclear supernatant from oleate-induced cells from both wild type and *yor180c*Δ strains, and fractionated this supernatant by sucrose density gradient ultracentrifugation (Fig. 5). Following ultracentrifugation, fractions were drawn from the bottom of the gradient, and these were

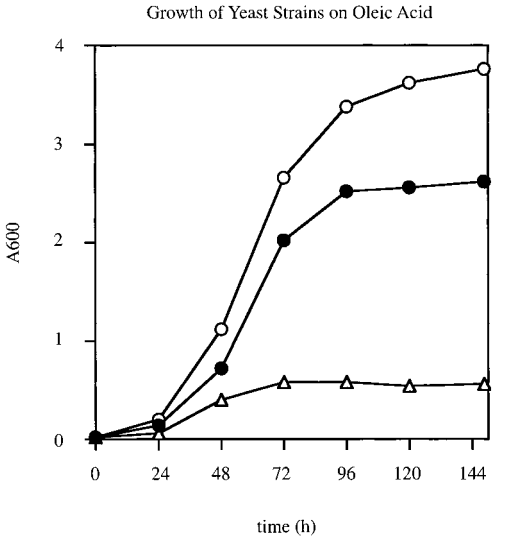

FIG. 4. *YOR180C* is required for normal yeast growth on oleic acid. The growth of *yor180c*Δ (filled circle), wild-type (open circle), and *pex8*Δ (open triangle) cells on oleic acid was monitored spectrophotometrically over a period of 150 h. The *yor180c*Δ cells grew more slowly than did wild type, indicating that *YOR180C* is required for normal growth on oleic acid.

assayed for the peroxisomal marker enzyme, catalase, and the mitochondrial marker enzyme, fumarase. During the course of these experiments, we also assayed both gradients for Δ^3,Δ^2-enoyl-CoA isomerase activity. In *S. cerevisiae* cells, all Δ^3,Δ^2-enoyl-CoA isomerase activity is encoded by the *ECI1* gene and the resulting enzyme, Eci1p, is localized exclusively to peroxisomes (Fig. 5A) (9). Surprisingly, in the *yor180c*Δ strain we found that Eci1p was distributed throughout many non-peroxisomal fractions despite the fact that this strain is not deficient in peroxisome biogenesis (Fig. 5B). Thus, it appeared that Yor180Cp is essential for normal subcellular localization of Eci1p in *S. cerevisiae.*

One prediction arising from this observation is that Yor180Cp and Eci1p might physically associate with one another. We tested this hypothesis using the yeast two-hybrid system. Vectors designed to express Yor180Cp in fusion with the Gal4p DNA binding domain (BD-Yor180Cp) and Eci1p in fusion with the Gal4p transactivation domain (AD-Eci1p) were constructed. These were used to test for physical interaction between Yor180Cp and Eci1p in a two-hybrid reporter strain, MaV99, that carries a chromosomal copy of a Gal4p-responsive β-galactosidase gene. β-galactosidase activity was then assayed in cell homogenates of MaV99 derivatives coexpressing BD-Yor180Cp and AD-Eci1p, as well as control strains coexpressing BD-Yor180Cp with AD and BD with AD-Eci1p, respectively. As shown in Table I, the level of β-galactosidase specific activity in the strain expressing both fusion partners was approximately 13-fold greater than in the control strains. This suggested that Yor180Cp and Eci1p are capable of forming a stable complex in *S. cerevisiae* cells.

DISCUSSION

In this study we provide a preliminary characterization of Yor180Cp, a novel peroxisomal protein that

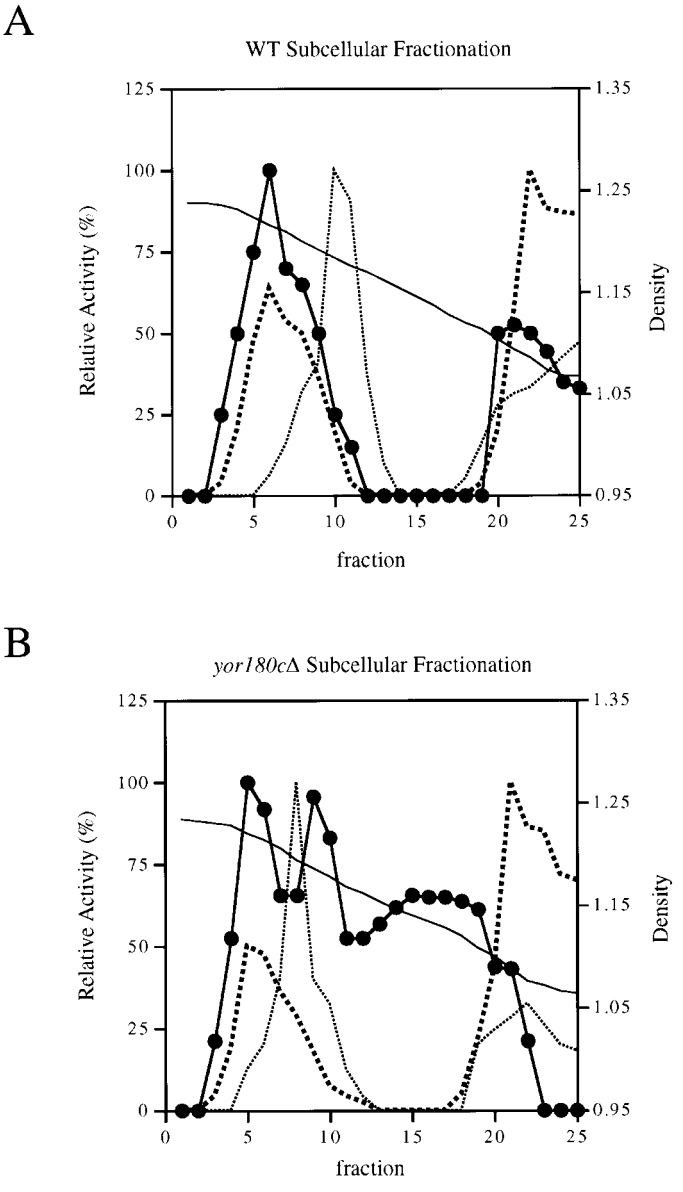

FIG. 5. *YOR180C* is essential for efficient import of Eci1p into peroxisomes. (A and B) Post-nuclear supernatants from wild-type and *yor180c*Δ cells, respectively, were fractionated by sucrose density gradient ultracentrifugation. Each fraction was assayed for Δ^3,Δ^2-enoyl-CoA isomerase (filled circle), catalase (dashed line), fumarase (dotted line), and density (solid line). Note the aberrant distribution of Δ^3,Δ^2-enoyl-CoA isomerase (Eci1p) in the *yor180c*Δ cells.

TABLE I

β-Galactosidase Activities in Cell Homogenates of Two-Hybrid Reporter Strains for Yor180Cp and Eci1p

Strain	BD-Yor180Cp AD-Eci1p	BD-Yor180Cp AD-	BD- AD-Eci1p	MaV99
β-galactosidase[1]	115	3.5	8.9	0

[1] Specific activity is reported in μU/mg protein ($\times 10^3$).

plays an accessory role in fatty acid utilization by *S. cerevisiae*. Removal of *YOR180C* from the yeast genome resulted in a mild but significant defect in yeast growth on fatty acids, and also resulted in a distinct mislocalization of Δ^3,Δ^2-enoyl-CoA isomerase (Eci1p) to non-peroxisomal locations within the cell. In addition, we observed that Yor180Cp is capable of physically associating with Eci1p in vivo. Taken together, these results are consistent with several possible functions for Yor180Cp in yeast peroxisomal fatty acid metabolism. First, the partial oleate-growth phenotype of the *yor180c*Δ strain is similar to that observed for the *sps19*Δ (6) and *idp3*Δ (8) mutants. These genes encode peroxisomal forms of 2,4-dienoyl-CoA reductase and $NADP^+$-dependent isocitrate dehydrogenase, respectively. These enzymes are auxiliary components of the unsaturated fatty acid oxidation pathways (26) and are involved in the $NADP^+$-dependent shunt of 2,5-dienoyl-CoA metabolism (27). This $NADP^+$-dependent shunt also requires the enzyme $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase (27,28) and, interestingly, this is the only known auxiliary enzyme of unsaturated fatty acid oxidation remaining to be identified in *S. cerevisiae* (9). Thus, it is possible that *YOR180C* encodes the yeast peroxisomal $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase. This idea is strengthened by the observation that Yor180Cp and Eci1p are the most similar proteins to each other in this organism, which might be expected considering the chemical similarity between the $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA and Δ^3,Δ^2-enoyl-CoA isomerase-catalyzed reactions.

We tested Yor180Cp directly for $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase activity but were unable to detect this activity with purified Yor180Cp. However, it should be noted that we were also unable to detect this activity in *S. cerevisiae* cell lysates or in purified yeast peroxisomes. Given that Yor180Cp can physically associate with Eci1p and that loss of *YOR180C* results in a mislocalization of Eci1p, a second possible role of Yor180Cp may be to chaperone Eci1p for efficient peroxisomal import. The presence of atypical PTS-1 signals on both proteins (His-Arg-Leu-COOH for Eci1p (9)) raises questions as to whether a Yor180Cp-Eci1p oligomer might be more efficiently targeted to peroxisomes than either protein individually. It is interesting to note that the mild oleate-growth phenotype displayed by the *yor180c*Δ strain correlates well with the partial loss of Eci1p localization to peroxisomes. Given that peroxisomal Eci1p is essential for yeast growth on oleic acid (9,10), this second role for Yor180Cp remains formally possible. Thus, further experimentation is needed to discriminate between these roles for peroxisomal Yor180Cp, as well as to address the possibility that a Yor180Cp-Eci1p oligomer may constitute the $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase of yeast.

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