Synthesis of Polyhydroxyalkanoate in the Peroxisome of *Saccharomyces cerevisiae* by Using Intermediates of Fatty Acid β-Oxidation

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Medium-chain-length polyhydroxyalkanoates (PHAs) are polyesters having properties of biodegradable thermoplastics and elastomers that are naturally produced by a variety of pseudomonads. *Saccharomyces cerevisiae* was transformed with the *Pseudomonas aeruginosa* PHAC1 synthase modified for peroxisome targeting by the addition of the carboxyl 34 amino acids from the *Brassica napus* isocitrate lyase. The PHAC1 gene was put under the control of the promoter of the catalase A gene. PHA synthase expression and PHA accumulation were found in recombinant *S. cerevisiae* growing in media containing fatty acids. PHA containing even-chain monomers from 6 to 14 carbons was found in recombinant yeast grown on oleic acid, while odd-chain monomers from 5 to 15 carbons were found in PHA from yeast grown on heptadecenoic acid. The maximum amount of PHA accumulated was 0.45% of the dry weight. Transmission electron microscopy of recombinant yeast grown on oleic acid revealed the presence of numerous PHA inclusions found within membrane-bound organelles. Together, these data show that *S. cerevisiae* expressing a peroxisomal PHA synthase produces PHA in the peroxisome using the 3-hydroxyacyl coenzyme A intermediates of the β-oxidation of fatty acids present in the media. *S. cerevisiae* can thus be used as a powerful model system to learn how fatty acid metabolism can be modified in order to synthesize high amounts of PHA in eukaryotes, including plants.

Polyhydroxyalkanoate (PHA) is a family of polyesters composed primarily of R-3-hydroxyalkanoic acids (2, 30, 40, 41). PHA is synthesized as intracellular inclusions by a wide variety of bacteria, including gram-positive and gram-negative species as well as some phototrophic bacteria, and is used as a carbon and electron sink. PHAs have been broadly defined in two main classes, namely, short-chain-length PHA (SCL-PHA) and medium-chain-length PHA (MCL-PHA). Whereas SCL-PHA typically harbors monomers of 3-hydroxy acids from 3 to 5 carbons, MCL-PHA contains 3-hydroxy acids from 6 to 16 carbons in length (2, 40). The best-characterized SCL-PHA is polyhydroxybutyrate (PHB), a homopolymer of 3-hydroxybutyric acid. In *Ralstonia eutropha*, PHB accumulates up to 80% of the dry weight (dwt), with inclusions being typically 0.2 to 1 μm in diameter. MCL-PHAs are typically synthesized by pseudomonads, such as *Pseudomonas oleovorans* and *Pseudomonas aeruginosa*.

Industrial interest in PHAs arises largely from their properties as thermoplastics and elastomers (2, 30, 40). Furthermore, numerous bacteria and fungi can hydrolyze PHAs to monomers and oligomers, which are further metabolized as a carbon source (2). PHAs are thus attractive as a source of renewable and biodegradable polyesters. PHB is a highly crystalline polymer with rather poor physical properties, being relatively stiff and brittle and degrading at temperatures slightly above its melting point (5). Incorporation of longer-chain monomers

into the polymer, to form the copolymer poly(hydroxybutyrate-cohydroxyvalerate) [P(HB-HV)], reduces the crystallinity and melting point of the polymer, leading to improvement in the flexibility, strength, and processing of the polymer (5).

In contrast to SCL-PHAs, which are regarded as thermoplastics, MCL-PHAs have low crystallinity and melting points and are generally regarded as elastomers (15). There exist two main pathways for the synthesis of MCL-PHAs in bacteria. In bacteria such as *P. oleovorans*, MCL-PHA accumulates when cells are grown on alkanoic acids as the carbon source (2, 40, 41). The nature of the PHA produced is related to the substrate used for growth and is typically composed of monomers that are $2n$ ($n \geq 0$) carbons shorter than the substrate. For example, growth of *P. oleovorans* on octanoate generates a PHA polymer containing 89 mol% 3-hydroxyoctanoic acid and 11 mol% 3-hydroxyhexanoic acid, whereas growth on dodecanoate generates PHA containing 31 mol% 3-hydroxydodecanoic acid, 36 mol% 3-hydroxydecanoic acid, 31 mol% 3-hydroxyoctanoic acid, and 2 mol% 3-hydroxyhexanoic acid (22). These studies have indicated that MCL-PHAs are synthesized by the PHA synthase from 3-hydroxyacyl coenzyme A (CoA) intermediates generated by the β-oxidation of alkanoic acids. A second pathway exists in some bacteria, such as *Pseudomonas putida*, that can synthesize MCL-PHA from glucose using intermediates of fatty acid biosynthesis (16, 20, 35, 43). The *phaG* gene that encodes a 3-hydroxyacyl-acyl carrier protein–CoA transferase provides the metabolic link between fatty acid biosynthesis and MCL-PHA synthesis (9, 32).

The main limitation for the use of PHAs as a commodity polymer is the high cost of producing PHA by bacterial fermentation relative to the cost of petroleum-derived plastics

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(27, 30). In this perspective, synthesis of PHAs in crop plants has been seen as an attractive alternative for the commercial production of large amounts of PHA at low cost (27, 28). Synthesis of SCL-PHAs has been demonstrated in a number of plants (28). Synthesis of PHB from acetyl-CoA was first shown in the cytoplasm and plastids of *Arabidopsis thaliana* cells (26, 29) and later in the peroxisomes of maize culture cells (14). Accumulation of PHB up to 40% dwt has recently been demonstrated in *A. thaliana* (4) as well as the synthesis of the copolymer P(HB-HV) in the seed leukoplasts of *A. thaliana* and *Brassica napus* (39). Furthermore, synthesis of PHB has also been demonstrated in the cytoplasm of *S. cerevisiae* (23) and in transgenic insect cells (45) expressing the PHB synthase from *R. eutropha*.

Synthesis of MCL-PHA in plants has been demonstrated in transgenic *A. thaliana* expressing the PHA synthase from *P. aeruginosa* in the peroxisome (24, 25). In these plants, MCL-PHAs containing saturated and unsaturated 3-hydroxyalkanoic acids ranging from 6 to 16 carbons were synthesized using intermediates of the β-oxidation of fatty acid (24, 25). The maximal amount of PHA synthesized in this system is low, at approximately 0.4 to 0.6% dwt, indicating the need for further modifications of fatty acid metabolic pathways before plants can be used for commercial production of MCL-PHAs.

We have sought to develop a more amenable eukaryotic system to understand how to manipulate the pathways involved in the degradation of fatty acids to increase the amount of MCL-PHA synthesized in peroxisomes. We now report the development of *Saccharomyces cerevisiae* for the synthesis of MCL-PHA in peroxisomes using intermediates of the β-oxidation of fatty acids.

MATERIALS AND METHODS

Strains and culture conditions. Plasmids were maintained and propagated in *Escherichia coli* DH5α according to Sambrook et al. (36). *S. cerevisiae* diploid strain INVSc1 (*his3D1 leu2 trp1-289 ura3-52*) was obtained from Invitrogen (Groningen, The Netherlands). *S. cerevisiae* harboring the PHA synthase gene was maintained in leucine-deficient media (0.67% yeast nitrogen base without amino acids [Difco, Detroit, Mich.], 0.5% ammonium sulfate, 2% glucose, and 0.4 g of leucine dropout supplement [Clontech, Palo Alto, Calif.]/liter). For PHA production, a stationary-phase culture was harvested by centrifugation and cells were washed once in water and resuspended at a 1:10 dilution in fresh leucine-deficient media containing 0.1% or no glucose as well as detergent (either Tween 80 or Pluronic-127 [Sigma, St. Louis, Mo.]) and fatty acid (oleic acid or heptadecenoic acid). Cells were grown for an additional 1 to 6 days before harvest of the cells for PHA analysis. The pH of the growth media with or without fatty acids was 6.0.

DNA constructs. The plasmid pYE352-PHA was constructed from the plasmid pYE352-CTA1 containing the *S. cerevisiae* catalase gene with its promoter and terminator sequences (10). The catalase-coding region of pYE352-CTA1 was removed by a *Sac*I-*Xho*I digestion, and the vector was made blunt ended by using T4 DNA polymerase. The PHA synthase from *P. aeruginosa* modified for peroxisomal targeting by the addition, at the carboxy end, of the last 34 amino acids of the *B. napus* isocitrate lyase, was obtained from the plasmid pART7-PhaC1-ICL (25). The plasmid pART7-PhaC1-ICL was digested by *Eco*RI-*Xba*I, and the fragment harboring the modified PHA synthase was made blunt ended by T4 DNA polymerase and was ligated to the blunt-ended pYE352-CTA1 vector to create pYE352-PHA. The gene expression cassette containing the CTA1 promoter-PHA synthase-CTA1 terminator was excised from pYE352-PHA by a partial *Eco*RI digestion and was cloned into the *Eco*RI site of the integrative shuttle vector Yiplac128 (12), giving the plasmid Yiplac128-PHA. This plasmid was linearized by digestion with *Cla*I and transferred into the *S. cerevisiae* strain INVSc1 by the lithium acetate procedure. Transformants were recovered on media without leucine.

Western blot analysis. The equivalent of an optical density at 600 nm of 0.2 of *S. cerevisiae* cells was harvested by centrifugation in a 1.5-ml tube and was suspended in 6 μl of 2 N NaOH–5% (vol/vol) β-mercaptoethanol. The suspension was put on ice for 10 min before adding 7.8 μl of 10% (wt/vol) sodium dodecyl sulfate. The mixture was briefly vortexed and was left at room temperature for 20 min with occasional mixing. The resulting crude protein extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was blotted onto nitrocellulose membranes using a Trans-Blot electrophoretic cell (Bio-Rad, Richmond, Calif.). Free binding sites were saturated by incubation in blocking buffer (0.4 M NaCl, 2 mM KCl, 20 mM Tris-HCl, pH 7.4, 1% [vol/vol] Tween 40, and 5% [wt/vol] milk powder) for 1 h. The membranes were incubated for 2 h at room temperature in blocking buffer with anti-PHA synthase antibody. The antigen-antibody complexes were visualized with horseradish peroxidase-coupled goat anti-rabbit antibodies using the enhanced-chemiluminescence method (Amersham, Little Chalfont, United Kingdom).

Analysis of fatty acids and PHA. Fatty acids present in the growth media were determined by analysis of fatty acid methyl-esters by gas chromatography (GC). Briefly, media were harvested and cleared of cells by centrifugation at 5,000 × g for 10 min. The cleared solution was transferred to a fresh tube and lyophilized. The dried residues were suspended in a methanol solution containing 1 N HCl and were heated at 80°C for 2 h. The fatty acid methyl-esters were extracted with 0.5 to 1 ml of hexane and 1 ml of 0.9% (wt/vol) NaCl, and the organic phase was transferred to autoinjector vials. GC analysis was performed using a Hewlett Packard 5890 gas chromatograph that was equipped with a Supelco SP2330 glass capillary column and coupled to a flame ionization detector.

For PHA analysis, cells were harvested by centrifugation, washed twice in water, and lyophilized. The dried material was then weighed (approximately 15 to 30 mg) and transferred to a glass tube. The material was extracted four or five times with warm (65°C) methanol to remove lipids, free fatty acids, and acyl-CoA, including 3-hydroxyacyl–CoA, while PHA, which is insoluble in methanol, remains associated with the cells. After centrifugation and removal of the residual methanol, the material was suspended in 0.5 ml of chloroform to which 0.5 ml of methanol containing 3% sulfuric acid was added. The mixture was heated at 95°C for 4 h and cooled down on ice. One milliliter of 0.1% NaCl was added to each tube, and the mixture was vortexed vigorously and centrifuged at 5,000 × g for 5 min. The chloroform phase was harvested and dried over anhydrous MgCl₂. The methyl-esters of 3-hydroxy acids were identified and quantified by GC-mass spectrometry (GC-MS) using a Hewlett-Packard 5890 gas chromatograph (HP-5MS column) coupled to a Hewlett-Packard 5972 mass spectrometer. Analysis by GC-MS was made utilizing the ion-selective mode (mass-to-charge ratio of 103). Identification of monomers present in plant PHA was facilitated by the use of commercial 3-hydroxy acid standards and purified bacterial PHAs. In one experiment, lyophilized cells were extracted with methanol in a Soxhlet apparatus for 24 h followed by PHA extraction with chloroform for 24 h. The PHA-containing chloroform was concentrated using a Rotovapor and extracted once with water to remove residual solid particles. PHA was precipitated by the addition of 10 volumes of cold methanol and was subsequently washed by two cycles of chloroform solubilization and methanol precipitation. PHA dissolved in chloroform was analyzed by GC-MS as described above. The composition of the PHA isolated after Soxhlet extraction was similar to the PHA analyzed following esterification of the methanol-washed cells in chloroform.

Electron microscopy. Cells were fixed for 4 h at room temperature in 4% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, and 0.1% (wt/vol) Brij 35, followed by an overnight treatment in the same solution without Brij 35. The cells were then rinsed several times with 0.1 M sodium cacodylate, pH 7.2, transferred to 2% osmium tetroxide for either 8 h at room temperature or 16 h at 4°C, and were then transferred to 2% uranyl acetate in 10% ethanol for 40 min. Cells were dehydrated through a graded series of ethanol with a final treatment in propylene oxide. Cells were embedded in Epon/Araldite resin and were polymerized for 3 days at 70°C. The blocks were cut with a Diatome diamond knife on a Reichret ultracut S microtome. Fine sections of 50 nm were placed on Formvar-coated copper grids, contrasted with a 2% aqueous solution of uranyl acetate for 6 min followed by lead citrate for 6 min. The grids were examined with a Philips Biotwin CM100 (Lab6 filament) transmission electron microscope.

RESULTS

Expression of the *P. aeruginosa* PHA synthase in recombinant yeast. Previous studies had shown that the PHA synthase from *P. aeruginosa* could be targeted to plant peroxisomes by modifying the carboxy end of the bacterial protein by the
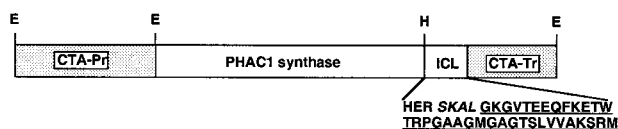
FIG. 1. DNA construct used to express the PHAC1 synthase of *P. aeruginosa* in *S. cerevisiae*. Only the portion of the construct containing the gene (open box) and regulatory elements (shaded box) is shown. The partial amino acid sequence of the C terminus of the PHA synthase-isocitrate lyase (ICL) fusion is indicated using the one-letter symbols, with the amino acids derived from the PHAC1 protein given in plain letters, novel amino acids created at the fusion junction italicized, and the last 34 amino acids derived from the *B. napus* isocitrate lyase underlined. CTA-Pr, CTA1 promoter; CTA-Tr, CTA1 terminator; E, *Eco*RI; H, *Hin*dIII.

TABLE 1. Synthesis of PHA in recombinant *S. cerevisiae* grown in various media

Treatment	PHA % (wt/dwt)
0.5% Tween 80	0.020 ± 0.004
0.5% Tween 80–0.1% oleic acid	0.20 ± 0.03
0.5% Tween 80–0.1% oleic acid–0.1% glucose	0.35 ± 0.03
2% Pluronic-127	<0.01
2% Pluronic-127–0.1% oleic acid–0.1% glucose	0.31 ± 0.03

addition of the last 34 amino acids of the peroxisomal protein isocitrate lyase from *B. napus* (25). This protein harbors the carboxy-terminal tripeptide ARM, a peroxisomal signal that was shown to be effective in targeting foreign proteins to peroxisomes in both plants and yeast (1, 44). The same modified PHA synthase was thus expressed in *S. cerevisiae*. The structure of the gene expression cassette is shown in Fig. 1. The modified PHA synthase was put under the control of the promoter and transcription terminator of the yeast catalase gene CTA1 (10). This promoter allows strong expression of genes in yeast grown in media containing fatty acids as the carbon source (19, 38). The expression cassette was cloned into the integrative yeast shuttle vector Yiplac128 for transformation into *S. cerevisiae*.

Western blot analysis of recombinant PHA synthase. Recombinant yeast transformed with Yiplac128-PHA was tested for expression of the PHA synthase by Western blot analysis using an anti-PHA synthase serum (Fig. 2). In these experiments, yeast grown to stationary phase in media containing 2% glucose was washed in water and resuspended at a 1:10 dilution in leucine-deficient media supplemented with glucose, oleic acid, and/or the detergent Pluronic-127 or Tween 80. While Pluronic-127 is a polyoxyethylene polymer containing no fatty acids, Tween 80 is a detergent containing approximately 20% oleic acid by weight that is esterified to the polyoxyethylene-sorbitan backbone. These detergents were used to insure solubilization of the free oleic acid added to the media. While no expression of the PHA synthase was detected in transformed cells grown for 24 h in media supplemented with 1% glucose, expression of the 65-kDa PHA synthase was readily detected in cells grown for 72 h in media supplemented with only 0.1% glucose or in media containing 0.1% glucose and either 0.5%

Tween 80, 2% Pluronic-127, 0.5% Tween 80 with 0.1% oleic acid, or 2% Pluronic-127 with 0.1% oleic acid (Fig. 2).

Production of MCL-PHA. Cells grown to stationary phase in media containing 2% glucose were shifted to media containing various fatty acids and detergents with or without 0.1% glucose and were grown for an additional 4 days before being harvested for PHA analysis (Table 1). Yeast grown on 0.5% Tween 80 produced only 0.02% (dwt) PHA, while addition of 0.1% oleic acid to media containing 0.5% Tween 80 led to a 10-fold increase of PHA to 0.2% (dwt). Since the PHA synthase is well expressed in yeast grown in media containing either Tween 80 alone or Tween 80 supplemented with free oleic acid (Fig. 2), these data indicate that free oleic acid is a better substrate for PHA synthesis in yeast than is the esterified oleic acid found in Tween 80. Addition of 0.1% glucose to media containing 0.5% Tween 80 and 0.1% oleic acid led to an increase of PHA to 0.35% (dwt). Recombinant yeast grown in media containing 0.1% glucose and 2% Pluronic-127 shows less than 0.01% PHA (Table 1), similar to cells grown in media containing only 0.1% or 2% glucose (data not shown). In contrast, cells grown in media supplemented with 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid produced 0.31% (dwt) PHA. No PHA could be detected in cells that were transformed with the control vector pYE352-CTA1 (which harbors the peroxisomal catalase gene) and were grown in media containing oleic acid (Fig. 3). Together, these data show that PHA synthesis in recombinant yeast is dependent on the presence of both a PHA synthase and an external source of fatty acids.

The amount of PHA in cells, as well as concentrations of oleic acid and glucose in the media, were monitored over 6 days after the shift of cells to media containing 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid (Fig. 4). The amount of glucose in the media was below detection (<0.002%) after 24 h, while oleic acid decreased only slightly to 0.09%. PHA accumulated in cells until day 5, in parallel with the decrease in the amount of free oleic acid in the media. At days 5 and 6, PHA was found in *S. cerevisiae* at 0.43 to 0.45% (dwt).

PHA inclusions detected by electron microscopy. Wild-type and recombinant cells grown for 3 days in media containing oleic acid were analyzed by transmission electron microscopy (TEM). While both types of cells showed the presence of numerous oil bodies (Fig. 5A and B), only recombinant cells producing PHA showed the presence of small electron-lucent
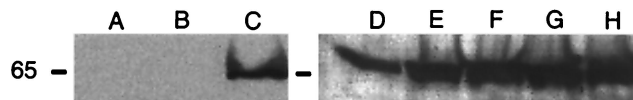

FIG. 2. Western blot analysis of PHA synthase expression in *S. cerevisiae*. Wild-type (A) or recombinant yeast transformed with the PHA synthase (B to H) was grown for 24 h in media containing 1% glucose (B) or 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid (A and C) or for 72 h in media containing 0.1% glucose (D); 0.1% glucose and 0.5% Tween 80 (E); 0.1% glucose and 2% Pluronic-127 (F); 0.1% glucose, 0.5% Tween 80, and 0.1% oleic acid (G); or 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid (H). Molecular mass marker (in kilodaltons) is indicated on the left.
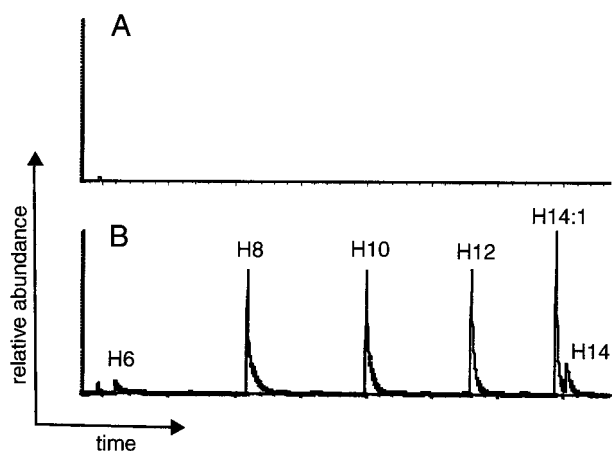
FIG. 3. GC-MS analysis of PHA produced in transgenic yeast expressing the PHA synthase. Yeast cells transformed with the control vector pYE352-CTA1 (A) or the plasmid pYE352-PHA harboring the PHA synthase gene (B) were grown for 3 days in media containing 2% Pluronic-127, 0.1% glucose, and 0.1% oleic acid, and the PHA was analyzed as described in Materials and Methods. Only ions with a mass-to-charge ratio of 103 are shown. The various 3-hydroxy acids are identified with the prefix H. The *y* axes in panels A and B are on the same scale.

inclusions within membrane-bound organelles (Fig. 5B to D). The apparent size of these inclusions is in the range of 0.1 to 0.2 μm in diameter. Both the size and general appearance of these inclusions, as seen by TEM, are very similar to PHA granules found in bacteria (2, 30) as well as in transgenic plants (25, 29), indicating that, similar to PHA synthesized in these other hosts, PHA produced in yeast accumulates in the form of inclusions.

Composition of MCL-PHA produced in yeast. In order to determine the influence of the carbon source on PHA monomer composition, recombinant yeast was grown for 4 days in media containing 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid or 0.1% heptadecenoic acid (17:1 Δ10*cis*). Table 2 shows that the PHA monomer composition is dependent on
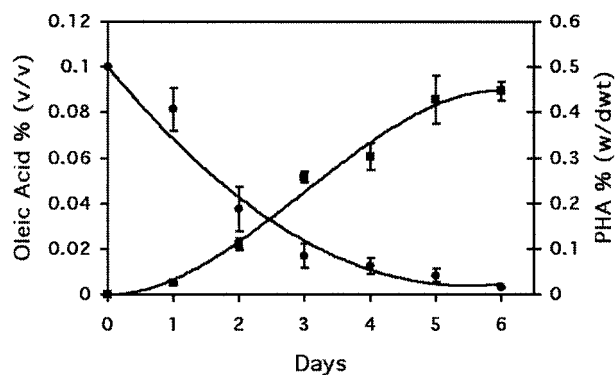

FIG. 4. Time course of the accumulation of PHA in *S. cerevisiae*. Recombinant yeast was used to inoculate media containing 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid. PHA content (■) in cells and the concentration of oleic acid (●) present in the media were monitored over 6 days. Values represent the mean and standard deviation of four measurements. w/dwt, weight/dwt; v/v, vol/vol.

the nature of the external fatty acids and on the range of 3-hydroxyacyl–CoA intermediates generated by the degradation of the external fatty acid by the peroxisomal β-oxidation pathway. PHA synthesized in yeast growing on oleic acid (18:1 Δ9*cis*) contains even-chain 3-hydroxy acid monomers from 6 to 14 carbons in length. The presence of both 3-hydroxytetradecanoic acid and 3-hydroxytetradecenoic acid agrees with the generation of the corresponding acyl-CoA by the β-oxidation of fatty acids having a *cis*-unsaturated bond at an odd-numbered carbon (13, 17). Similarly, growth of recombinant yeast in media containing 17:1 Δ10*cis* gave a PHA containing odd-chain monomers ranging from 5 to 15 carbons, with two monomers being unsaturated (Table 2). Together, these data show that external fatty acids are imported into cells and degraded via the peroxisomal β-oxidation cycle and that 3-hydroxyacyl–CoAs generated by the β-oxidation cycle are used for the synthesis of MCL-PHA.

DISCUSSION

Expression in *S. cerevisiae* of a PHA synthase from *P. aeruginosa*, modified at the carboxy end by the addition of a peroxisome targeting signal, was shown to lead to MCL-PHA synthesis in yeast growing in media containing fatty acid. Expression of MCL-PHA was dependent on the promoter of the CTA1 gene encoding the peroxisomal catalase A. This gene was shown previously to be repressed by glucose and activated by fatty acids (19, 38). In consequence, the PHA synthase was found to be expressed in cell cultures that had depleted the glucose initially present in the media, as well as in cells growing in media containing fatty acids.

Feeding experiments with oleic acid or heptadecenoic acid added to the growth media clearly indicate that the monomers used in the synthesis of MCL-PHA in yeast are derived from the β-oxidation of the external fatty acids. Thus, addition of heptadecenoic acid leads to the synthesis of MCL-PHA containing odd-chain monomers, while addition of oleic acid leads to the synthesis of a PHA containing even-chain monomers. Furthermore, for each of these fatty acids, the monomers found in PHA correspond to the 3-hydroxyacyl–CoAs generated by the degradation of the external fatty acid through peroxisomal β-oxidation. Thus, degradation of the fatty acid 17:1 Δ10*cis*, according to the pathway for the degradation of fatty acids having a *cis* double bond at an even-numbered carbon (13), is expected to lead to the production of the three unsaturated 3-hydroxyacyl–CoAs, namely, H17:1 (the prefix H refers to the 3-hydroxy moiety), H15:1, and H13:1, and of five saturated 3-hydroxyacyl–CoAs, namely, H11, H9, H7, H5, and H3. All these 3-hydroxy acids are found in the PHA synthesized in *S. cerevisiae* grown in 17:1 Δ10*cis*, except for H17:1 and H3, which fall outside the range of monomers generally accepted by the *P. aeruginosa* PHA synthase. Incorporation of 3-hydroxypentanoic acid in the yeast PHA is interesting, since PHA synthesized by *P. aeruginosa*, as well as other by pseudomonads producing MCL-PHAs, typically includes only monomers between 6 and 16 carbons (40). It has been previously reported that expression of a PHA synthase in a heterologous host may lead to the incorporation into PHA of a range of monomers broader than that normally found in the polymer of the native host (3, 6, 21). For example, while *Chromobacterium*
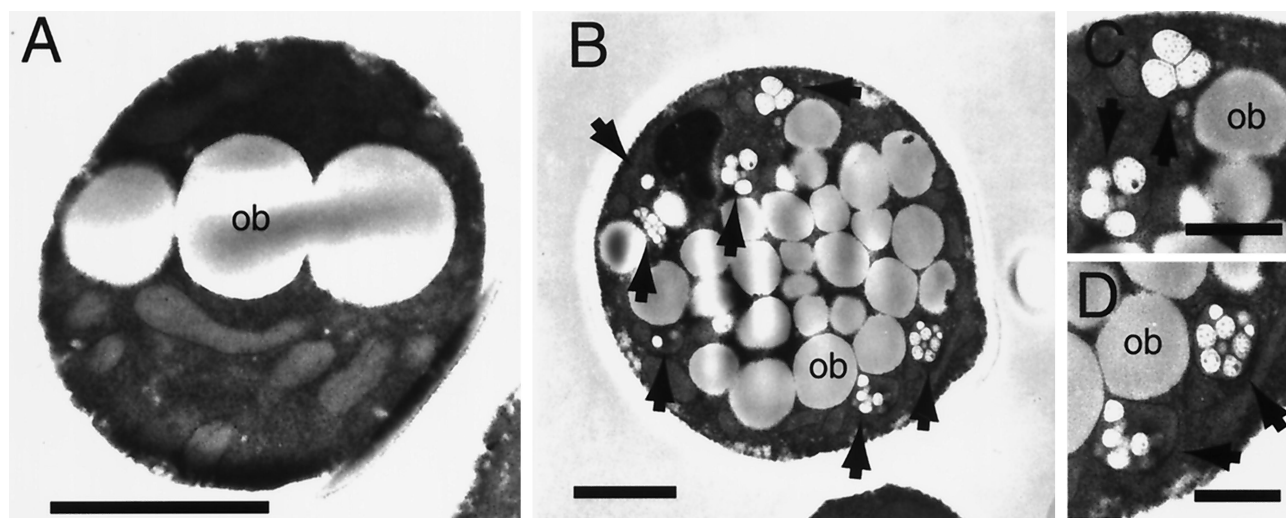
FIG. 5. Analysis of PHA inclusions in *S. cerevisiae*. The wild-type (A) or recombinant yeast expressing the PHA synthase gene (B to D) was used to inoculate media containing 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid and was grown for 4 days before being processed for TEM. Panels C and D are close-up views of panel B. Arrows indicate the presence of PHA inclusions within membrane-bound organelles. ob, oil body. Bars indicate 1 μm (A and B) and 0.5 μm (C and D).

violaceum growing on fatty acids produces a PHA containing only 3- or 4-carbon monomers, expression of the *C. violaceum* PHA synthase in *R. eutropha* leads to the synthesis of PHA containing in addition a 6-carbon monomer (21). These studies demonstrate that the PHA monomer composition is not only dependent on the substrate specificity of the PHA synthase but also on the metabolic environment of the host organism. Thus, in contrast to *P. aeruginosa*, the β-oxidation cycle of yeast allows the inclusion of 3-hydroxypentanoic acid into PHA.

Growth of recombinant yeast on oleic acid leads to the synthesis of PHA containing even-chain monomers from 6 to 14 carbons that are generated by the degradation of this fatty acid via the peroxisomal β-oxidation cycle, namely, H14:1, H14, H12, H10, H8, and H6. No 16-carbon 3-hydroxy acids were detectable in the yeast PHA, even though expression of the same PHA synthase in the peroxisome of the plant *A. thaliana* led to the incorporation of saturated and unsaturated 16-carbon monomers in the PHA (25). It is again likely that differences in the in vivo concentration or availability of the 3-hydroxyhexadecanoyl–CoA intermediates between *S. cerevisiae* and *A. thaliana* may influence the incorporation of 16-carbon monomers into PHA.

PHA production in yeast is accompanied by the appearance of electron-lucent inclusions within membrane-bound organelles. The size and general appearance of these inclusions are very similar to PHA granules found in bacteria or plants accumulating PHA. TEM, by itself, cannot unambiguously

identify the organelle containing the PHA inclusions as being peroxisomes. However, all results obtained strongly support the notion that PHA granules are found within the peroxisomes. First, PHA synthesized in yeast is clearly derived from intermediates of β-oxidation of the fatty acids added to the media. Furthermore, we have recently found that expression of the modified PHA synthase in the *fox1* mutant, deficient in the peroxisomal β-oxidation enzyme acyl-CoA oxidase, does not produce MCL-PHA (data not shown). These data show that MCL-PHA synthesis depends directly on substrates generated by the peroxisomal β-oxidation cycle. Second, the PHA synthase contains a peroxisomal targeting sequence that has been clearly shown to direct foreign proteins to the peroxisome (1, 44). Finally, β-oxidation is found to occur only in the peroxisome in *S. cerevisiae*.

In bacteria and plants, PHAs are composed of the R isomer of 3-hydroxyalkanoic acids due to the stereospecificity of the PHA synthase, which accepts only R-3-hydroxyacyl–CoAs (15). Since the core β-oxidation cycle of bacteria, mammals, and plants generates mainly the S isomer of 3-hydroxyacyl–CoAs from the hydration of enoyl-CoAs by the enoyl-CoA hydratase I (17, 37), synthesis of MCL-PHAs in these organisms implicates the presence of enzymes which can convert intermediates of β-oxidation to R-3-hydroxyacyl–CoAs. In several bacteria synthesizing MCL-PHAs from alkanoic acids, an R-specific enoyl-CoA hydratase II has been identified which converts the β-oxidation intermediate *trans*-2-enoyl–CoAs to R-3-hydroxya-

TABLE 2. Monomer composition of PHA synthesized in *S. cerevisiae*

Fatty acid[b]	PHA monomer composition (mol%)[a] for:											
	H5	H6	H7	H8	H9	H10	H11	H12	H13:1	H14:1	H14	H15:1
18:1 Δ9*cis*		21		46		19		10		3.8	0.9	
17:1 Δ10*cis*	9.4		40		22		16		11			1.7

[a] 3-Hydroxy acid monomers are denoted by the prefix H.
[b] Cells were grown in a leucine-deficient medium supplemented with 0.1% glucose, 2% Pluronic-127, and 0.1% fatty acids.

cyl–CoAs (11, 33). Furthermore, a 3-ketoacyl–ACP reductase has also been identified in *E. coli* and *P. aeruginosa* which can contribute to the synthesis of R-3-hydroxyacyl–CoAs from 3-ketoacyl–CoA (34, 42). In plants, a range of R-3-hydroxyacyl–CoAs is thought to be generated by either a monofunctional enoyl-CoA hydratase II or the 3-hydroxyacyl–CoA epimerase activity found within the β-oxidation multifunctional protein, which also harbors an enoyl-CoA hydratase I and a 3-hydroxyacyl–CoA dehydrogenase (8, 31).

In contrast to bacteria, plants, and animals, many fungi, including *S. cerevisiae* and *Candida tropicalis*, have a β-oxidation cycle that normally occurs through R-3-hydroxyacyl–CoA intermediates. This is because these organisms have only an R-specific enoyl-CoA hydratase II instead of an S-specific enoyl-CoA hydratase I (18). It was thus expected that *S. cerevisiae* would be a good host for the synthesis of MCL-PHAs from the intermediates of fatty acid β-oxidation. However, PHA synthesis in yeast is 1 or 2 orders of magnitude lower then MCL-PHA production in several pseudomonads. For example, growth of *P. putida* in media containing oleic acid gives an accumulation of 37% MCL-PHA (7), while in this study recombinant *S. cerevisiae* accumulates a maximum of 0.45% PHA. Although one should be cautious about comparing PHA synthesis in bacteria grown in fermentors and yeast cultures grown in shake flasks, these studies nevertheless indicate that PHA synthesis in yeast is suboptimal compared to bacterial production. This raises the interesting question of the biochemical basis of this difference.

Interestingly, although the isomers of 3-hydroxyacyl–CoA generated by the β-oxidation cycle in yeast and plants are different, the levels of PHA synthesized in the peroxisome of these two organisms are similar at 0.4% (dwt) (24, 25). These results indicate that the stereospecificity of the substrate generated by the core β-oxidation cycle may not be the only factor influencing PHA synthesis from β-oxidation intermediates. Although it is possible that some of the factors limiting MCL-PHA synthesis in yeast and plants may be different, the pathways of degradation of fatty acids is sufficiently similar between these organisms to consider using the power of yeast genetics as a tool to understand how more intermediates from the peroxisomal β-oxidation cycle can be channeled towards PHA and to apply this knowledge to the production of MCL-PHA in plants.

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