Increasing the Carbon Flux toward Synthesis of Short-Chain-Length–Medium-Chain-Length Polyhydroxyalkanoate in the Peroxisome of *Saccharomyces cerevisiae* through Modification of the β-Oxidation Cycle

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Short-chain-length–medium-chain-length polyhydroxyalkanoates were synthesized in *Saccharomyces cerevisiae* from intermediates of the β-oxidation cycle by expressing the polyhydroxyalkanoate synthases from *Aeromonas caviae* and *Ralstonia eutropha* in the peroxisomes. The quantity of polymer produced was increased by using a mutant of the β-oxidation-associated multifunctional enzyme with low dehydrogenase activity toward *R*-3-hydroxybutyryl coenzyme A.

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxy acids naturally synthesized as intracellular inclusions by a wide variety of bacteria (11, 14, 15). These polymers have attracted considerable attention because of their properties as biodegradable plastics and elastomers. PHAs can be subdivided into three main groups: namely, short-chain-length PHAs (SCL PHAs) containing mainly 3-hydroxy acids ranging from 3 to 5 carbons, medium-chain-length PHAs (MCL PHAs) containing 3-hydroxy acids ranging from 6 to 16 carbons, and the hybrid SCL-MCL PHAs containing 3-hydroxy acids from 4 to 12 carbons.

One of the main limitations for the use of PHAs as biodegradable plastics used in high-volume, low-value commodity products is their relatively high production cost through bacterial fermentation relative to petroleum-derived plastics such as polypropylene. Synthesis of PHAs has been demonstrated in several genetically engineered plants as well as in recombinant yeast (4, 8–10, 13). Synthesis of PHAs in crop plants has been seen as a promising alternative approach for their production since this PHA combines the properties of flexibility and toughness similar to those of polypropylene and desired for bulk commodity plastics (1). We have targeted two distinct PHA synthases to yeast peroxisomes to access the R-3-hydroxyacyl-CoA intermediates of the β-oxidation cycle and have examined the effects of the expression of variants of the β-oxidation multifunctional enzyme on the quantity and monomer composition of PHA synthesized.

The PHAC synthases from *Ralstonia eutropha* (PHACRe) and *Aeromonas caviae* (PHACAc), two bacteria known to produce SCL or SCL-MCL PHA, were modified at the N termini by using oligonucleotides to add the first 16 amino acids derived from the *S. cerevisiae* peroxisomal 3-ketothiolase protein (PTO1, or FOX3), which harbors a peroxisomal targeting sequence (PTS2) (3) (Fig. 1). Previous experiments have shown that these amino-terminal 16 amino acids are necessary and sufficient to target cytoplasmic proteins to the peroxisome (3). The chimeric genes were cloned into the yeast centromeric vector p416GPD, putting the genes under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, resulting in the constructs p415GPD::PTS2-PHACRe and p415GPD::PTS2-PHACAc (6). Mutants with mutations in the 3-hydroxyacyl-CoA dehydrogenase A and B domains of the *S. cerevisiae* multifunctional enzyme (MFE-2) encoded by the FOX2 gene have been previously described by Qin et al. (12). Briefly, the MFE-2(aΔ) mutant retains a broad activity towards short (C4)-, medium (C10)-, and long (C16)-chain R-3-hydroxyacyl-CoAs, while the MFE-2(bΔ) mutant shows highest activity with medium- and long-chain R-3-hydroxyacyl-CoAs and does not accept the short-chain R-3-hydroxybutyryl-CoA (12). The plasmid pYE352::ScMFE-2 containing the intact multifunctional gene from *S. cerevisiae*, as well as the plasmids pYE352::ScMFE-2(aΔ) and pYE352::ScMFE-2(bΔ) containing the mutated variants of the MFE-2 gene, have been previously described (12). All MFE-2 gene constructs were expressed in the vector pYE352, placing the genes under the control of the catalase A (CTA1) promoter and terminator (12). Plasmids harboring the various PHA synthases and MFE-2 constructs were transformed by the lithium acetate...
FIG. 1. DNA constructs used to express the PHA synthase of *R. eutropha* or *A. caviae* in *S. cerevisiae*. The 16 amino acids of the *S. cerevisiae* 3-ketoisooascorbic (FOX3) harboring the peroxisome targeting sequence (PTS2) are indicated in capital letters in the first open box. The cloning strategy used to make an in-frame fusion between PTS2 and either PHAC<sub>Re</sub> or PHAC<sub>Ac</sub> synthase (top and bottom lines of the second open box, respectively) created a histidine amino acid, indicated in italics. The promoter of the glyceraldehyde-3-phosphate dehydrogenase promoter (GPD-Pr) and terminator of the cytochrome c oxidase (CYC1-Tr) are indicated by shaded boxes. B, BamHI; H, HindIII. The figure was not drawn to scale.

procedure (2) into the *S. cerevisiae* mutant fox2Δ0 (YKRO099C::kanMX4) in the BY4742 background (MATa his3Δ1 leu2Δ0 his3Δ2 ura3Δ0) obtained from EUROSCARF (http://www.uni-frankfurt.de/ib15/mikro/euroscarf/index.html). For the synthesis of PHA, cells were grown in selective medium containing 0.1% (vol/vol) oleic acid, and the polymer was analyzed as previously described (9).

Expression of the PTS2-modified PHAC<sub>Re</sub> or PHAC<sub>Ac</sub> in the fox2Δ0 deletion mutant resulted in no PHA accumulation (data not shown), consistent with the requirement of a functional β-oxidation cycle for SCL-MCL PHA synthesis. Thus, fox2Δ0 cells expressing PHAC<sub>Re</sub> or PHAC<sub>Ac</sub> were retransformed with the plasmid pYE352::ScMFE-2, pYE352::ScMFE-2(Δ4), or pYE352::ScMFE-2(Δ6). The quantity and monomer composition of PHA synthesized in the various strains grown in media containing 0.1% oleic acid as the main carbon source are shown in Table 1.

Both the PHA quantity and monomer composition remained relatively unchanged in the strains fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2 and fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ). In contrast, strain fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2(Δ) accumulated considerably more PHA than the two other strains, increasing from 1.5 × 10<sup>-4</sup> g of PHA per g of cell (dry weight) for fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2 and fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) to 1.1 × 10<sup>-3</sup> g of PHA per g of cell (dry weight) for strain fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ). No significant differences were observed in the PHA monomer composition between cells expressing the wild type and the variant MFE-2. In parallel to the data obtained with the PHAC<sub>Re</sub> variant and monomer composition of the PHA produced in the fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2 and fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2(Δ) remained unchanged, while there was a 3.8-fold increase in the quantity of PHA in fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2(Δ), from 1.3 × 10<sup>-4</sup> g of PHA/g of cell (dry weight) to 5.0 × 10<sup>-4</sup> g of PHA/g of cell (dry weight). Furthermore, a small but significant decrease in the quantity of the 3-hydroxyhexanoic acid monomer is observed, going from 14 mol% in the fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2 and fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2(Δ) strains to 8 mol% for fox2Δ0/PHAC<sub>Ac</sub>/ScMFE-2(Δ).

It has been previously shown for MCL PHA synthesized in recombinant *S. cerevisiae* expressing the PHA synthase from *P. aeruginosa* (PHAC<sub>Pa</sub>) in the peroxisomes that coexpression of the MFE-2(Δ) variant resulted in an approximate twofold increase in the proportion of the 5- and 6-carbon monomers but had no impact on the quantity of PHA synthesized (5). The effect of the expression of the MFE-2(Δ) on PHA quantity and monomer composition can be explained by the potential impact of the expression of the MFE-2(Δ) on the R-3-hydroxyacyl-CoA pools. In vitro measurements of the k<sub>cat</sub> value of the MFE-2(Δ) revealed an undetectable dehydrogenase activity toward R-3-hydroxybutyryl-CoA, while k<sub>cat</sub> values toward R-3-hydroxydecanoyl-CoA and R-3-hydroxyhexadecanoyl-CoA were minimally changed compared to those of the wild type (12). Thus, expression of MFE-2(Δ) would result in a shift in the relative abundance of the various R-3-hydroxyacyl-CoAs, with the short-chain R-3-hydroxyacyl-CoAs being relatively more abundant compared to the medium- or long-chain R-3-hydroxyacyl-CoAs, and thus more available for their incorporation into PHA. In the case of coexpression of PHAC<sub>Pa</sub> with MFE-2(Δ), although the k<sub>cat</sub> values for R-3-hydroxyvaleryl-CoA and R-3-hydroxydecanoyl-CoA were not measured, the fact that the proportion of the 5- and 6-carbon monomers increased indicates that the affinity of the MFE-2(Δ) for these substrates is also probably reduced, and thus the availability of these intermediates for PHA synthesis increased. However, since the 5- and 6-carbon monomers represent only a small fraction of the monomers present in MCL PHA, the impact on the total amount of PHA is very limited. In contrast, in the case of the synthesis of SCL-MCL PHA from the coexpression of PHAC<sub>Re</sub> or PHAC<sub>Ac</sub> with MFE-2(Δ), the increased availability of short-chain R-3-hydroxyacyl-CoAs has a significant impact on PHA quantity, since monomers between 4 and 6 carbons form the entirety of the SCL-MCL PHA synthesized in these cells. The fact that a small but significant decrease in the proportion of the R-3-hydroxyhexanoic acid monomer is observed in cells expressing the PHAC<sub>Ac</sub> and MFE-2(Δ) indicates that the variant enzyme may retain some activity toward R-3-hydroxyhexanoyl-CoA, thus decreasing the relative abundance of R-3-hydroxyhexanoyl-CoA compared to R-3-hydroxybutyril-CoA.

In conclusion, this work demonstrates that improvement in the quantity of SCL-MCL PHA synthesized in *S. cerevisiae* peroxisomes from β-oxidation intermediates is possible through the use of a mutant MFE-2 enzyme having reduced dehydrogenase activity for short-chain R-3-hydroxyacyl-CoAs.

**TABLE 1.** Synthesis of SCL-MCL PHA in *S. cerevisiae* through coexpression of *R. eutropha* or *A. caviae* PHA synthase and variants of *S. cerevisiae* MFE-2

| Strain<sup>a</sup> | PHA quantity<sup>b</sup> | Monomer composition (mol%)<sup>c</sup> |
|------------------|-------------------------|---------------------------------------|
|                  |                        | H4 | H5 | H6 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2 | 1.5 ± 0.3 | 93 ± 2 | 6 ± 1 | 1.3 ± 0.4 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) | 1.5 ± 0.2 | 94 ± 1 | 5 ± 1 | 1.4 ± 0.3 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) | 11 ± 1 | 91 ± 1 | 7 ± 1 | 1.5 ± 0.2 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) | 13.0 ± 0.2 | 81 ± 2 | 4 ± 1 | 14 ± 1 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) | 8.0 ± 0.2 | 81 ± 2 | 4 ± 1 | 14 ± 1 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) | 14.0 ± 0.2 | 81 ± 1 | 4 ± 1 | 14 ± 1 |
| fox2Δ0/PHAC<sub>Re</sub>/ScMFE-2(Δ) | 5.0 ± 0.5 | 81 ± 5 | 6 ± 1 | 8.0 ± 0.5 |

<sup>a</sup> Cells were grown in media containing 0.1% oleic acid. PHA quantity is indicated as (gram weight of PHA/gram of cell dry weight) × 10<sup>-4</sup>. Values are averages ± standard deviation (n = 3).

<sup>b</sup> Strain: A strain containing PHA synthase from *R. eutropha* or *A. caviae* with MFE-2(Δ) enzyme.

<sup>c</sup> Cells expressing PTS2-modified PHAC<sub>Re</sub> or PHAC<sub>Ac</sub> and either the wild-type MFE-2 enzyme of *S. cerevisiae* or the mutated MFE-2(Δ) enzyme.
V.C.D.O. was a recipient of a bursary from the Commission Fédérale des Bourses pour Étudiants Étrangers, and I.M. was a recipient of a fellowship of the Fonds National Suisse de la Recherche Scientifique (83JS-067392). This research was also partially funded by the Université de Lausanne, by the Canton de Vaud, and by a grant from the Fonds National Suisse de la Recherche Scientifique (3100-061731).

We thank Y. Doi (RIKEN, Japan) for providing the PHA synthase from *A. caviae*, as well as Silvia Marchesini and Simon Goepfert (Université de Lausanne) for help with the gas chromatography-mass spectrometry.

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