***Saccharomyces cerevisiae* expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate**

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The polyhydroxybutyrate (PHB) synthase gene of the bacterium *Alcaligenes eutrophus* was used to construct a yeast plasmid which enabled expression of the functional synthase enzyme in *Saccharomyces cerevisiae*. Cells transformed with the synthase plasmid accumulated up to 0.5% of cell dry weight as PHB, with accumulation occurring in the stationary phase of batch growth. The identity of PHB in recombinant yeast cells was confirmed with ¹H-NMR spectra of chloroform-extracted cell material. In addition, freeze-fracture electron microscopy revealed cytoplasmic granules exhibiting plastic deformations characteristic for PHB. GC results indicated a low background level of PHB in the wild-type strain, but intact polymer could not be detected by ¹H-NMR. Formation of PHB in the recombinant strain implies the participation of native yeast enzymes in the synthesis of D-3-hydroxybutyryl-CoA (3-HB-CoA). Inhibition studies with cerulenin indicated that the fatty acid synthesis pathway is not involved in PHB precursor formation. Wild-type cell-free extracts showed D-3-HB-CoA dehydrogenase activity [150–200 nmol min⁻¹ (mg protein)⁻¹] and acetoacetyl-CoA thiolase activity [10–20 nmol min⁻¹ (mg protein)⁻¹], which together could synthesize monomer from acetyl-CoA. PHB accumulation was simultaneous with ethanol production, suggesting that PHB can act as an alternate electron sink in fermentative metabolism. We propose that PHB synthesis in recombinant yeast is catalysed by native cytoplasmic acetoacetyl-CoA thiolase, a native β -oxidation protein possessing D-3-HB-CoA dehydrogenase activity and heterologous PHB synthase.

Keywords: *Saccharomyces cerevisiae*, polyhydroxybutyrate, lipid metabolism, metabolic engineering

INTRODUCTION

Poly-3-hydroxybutyrate (PHB) is one of the polyhydroxyalkanoates synthesized by bacteria as carbon and energy storage materials (Steinbüchel, 1991). Over the past decade this material has received much attention as a biodegradable thermoplastic, although microbial production costs have limited its commercial exploitation. PHB synthesis is generally regarded as foreign to eukaryotic cells, although evidence exists that yeast and many other eukaryotic cells contain small amounts of low molecular mass PHBs which function as complexes with

polyphosphate in membrane transport (Reusch, 1989). Eukaryotic production of PHB through genetic engineering, especially in plants, is being pursued as a potentially inexpensive alternative to prokaryotic production (Nawrath *et al.*, 1994).

One of the most studied bacterial PHB pathways is that of *Alcaligenes eutrophus*, which involves three enzymes: 3-ketothiolase, acetoacetyl-CoA (AcAc-CoA) reductase (also called 3-hydroxybutyryl-CoA dehydrogenase), forming D-3-hydroxybutyryl-CoA (D-3HB-CoA), and PHB synthase, which in sequence catalyse the formation of PHB from acetyl-CoA (Ac-CoA) (Steinbüchel, 1991). Formation of PHB in our eukaryotic system, *Saccharomyces cerevisiae*, could be accomplished by synthesis of these bacterial enzymes and their proper compartmentation with respect to precursor and cofactor pools. PHB

Abbreviations: Ac-CoA, acetyl-CoA; AcAc-CoA, acetoacetyl-CoA; FAS, fatty acid synthetase; 3HB, 3-hydroxybutyrate; D-3HB-CoA, D-3-hydroxybutyryl-CoA; PHB, poly-3-hydroxybutyrate.

synthesis would interfere with primary metabolism by diverting carbon from the available Ac-CoA pool and by consuming reducing equivalents. The magnitude of the flux through this pathway would determine the effect on growth kinetics and physiology of a cell.

The ubiquitous nature of thiolases (Ac-CoA acetyltransferases) suggests that only reductase and synthase enzymes are necessary to complete the PHB pathway of *A. entrophus* in a new host organism. This has been found to be the case for plant cells (Poirier *et al.*, 1992; Nawrath *et al.*, 1994). Three thiolases are known to exist in *S. cerevisiae*, functioning in mitochondria, cytoplasm (Kornblatt & Rudney, 1971) and peroxisomes (Igual *et al.*, 1991). *S. cerevisiae* also possesses a peroxisomal D-3-hydroxyacyl-CoA dehydrogenase acting in the β -oxidation pathway (Hiltunen *et al.*, 1992). This dehydrogenase can catalyse conversion of AcAc-CoA to D-3HB-CoA, but to participate in PHB synthesis it must be present in the same cellular compartment as thiolase and synthase activities. We therefore expected that PHB synthesis in the yeast cytoplasm would require introduction of both reductase and synthase activities. We have found, however, that expression of the PHB synthase gene is sufficient for *S. cerevisiae* to accumulate PHB in the cytoplasm. This result implies that wild-type yeast synthesizes D-3HB-CoA, which could arise from intermediates in fatty acid synthesis or β -oxidation. Experimental results suggest that yeast transformed with a synthase plasmid form PHB via a pathway identical to that found in *A. entrophus*, with native enzymes providing both thiolase and reductase activities.

METHODS

Strains and media. For routine cloning of plasmids, *Escherichia coli* strain MC1061 was used (Meissner *et al.*, 1987). All *E. coli* plasmids and yeast shuttle vectors contained a β -lactamase gene and transformed *E. coli* was grown in LB medium or 2 $\times$ YGT medium (Maniatis *et al.*, 1982) supplemented with ampicillin (100 μ g ml⁻¹).

Saccharomyces cerevisiae strain D603 (MATa/MAT α *ura3-52 lys2-801 met his3 ade2-101 reg1-501*) was used as a host strain. This strain carries a mutation (*reg1*) that releases repression of galactose genes by glucose (Matsumoto *et al.*, 1983). Strain YPH500 (MAT α *ura3-52 lys2-801 ade2-101 trp1- Δ 63 his3- Δ 200 leu2- Δ 1*), lacking a *reg1* mutation, was also used. Yeast strains were grown in standard SD medium supplemented with appropriate amino acids and either glucose or galactose (Sherman *et al.*, 1979) or in an enriched version of SD medium (DaSilva, 1988) which allowed growth to higher cell densities. All yeast media contained initial sugar concentrations of 20 g l⁻¹. In some cases the specific enzyme inhibitors cerulenin or compactin (Sigma) were added to media.

Cell growth. Batch cultures were carried out in 21 LH bioreactors (LH Fermentation) with a working volume of 1.5 l. Cultures were maintained at a pH of 4.5 and a temperature of 30 °C. The agitation rate was 700 r.p.m. and air flow rate was 1 culture vol. min⁻¹. Dissolved oxygen did not drop below 90% over the course of batch growth. Shake flask cultures were carried out in 250 ml Erlenmeyer flasks containing 50 ml medium. The shake flasks were maintained at 30 °C in a Lab-line incubator-shaker running at 240 r.p.m.

Fig. 1. Diagram of plasmid pTL85. Details of construction are given in Methods. Note the relationship between translation start and the beginning of bacterial DNA. The linear map represents the PHB operon of *A. entrophus*.

Plasmid construction. Plasmid DNA was extracted from *E. coli* by alkaline lysis (Maniatis *et al.*, 1982). Restriction endonuclease and DNA modifying enzymes were obtained from Gibco or NEB and used according to manufacturers' specifications.

Plasmid pAet41 (Peoples & Sinskey, 1989), a gift from A. Sinskey, contains the entire PHB operon of *A. entrophus* cloned into an *E. coli* vector, and served as the source for PHB genes. Plasmid pLGSD5 (Guarente *et al.*, 1982) contains a *GAL10/CYC1* hybrid promoter and a convenient cloning site located several base pairs downstream of the ATG translation start codon, and thus represents a vector for making translational fusions with heterologous genes. Another useful element of this plasmid is a cassette containing the yeast 2 μ m plasmid origin of replication.

Plasmid pTL85 (Fig. 1) was constructed as follows: plasmid pMP69, carrying only the synthase from the PHB operon, was derived from pAet41 by deletion of *Bam*HI–*Bst*BI and *Stu*I–*Afl*II fragments, blunt-ending with Klenow, and religating. pMP76 was constructed by ligating a 1.8 kb *Hind*III–*Bam*HI fragment carrying the yeast *URA3* gene and *GAL10/CYC1* promoter sequence from pLGSD5 into a backbone of pMP69. An in-frame fusion of the synthase (*phbC*) gene with the promoter was then formed by deletion of a *Bam*HI–*Not*I fragment, blunt-ending with Klenow, and religating to form pTL77. Finally, a 2.2 kb *Eco*RI fragment from pLGSD5 harboring the yeast 2 μ m plasmid origin of replication was ligated into a backbone of pTL77 to get pTL85.

E. coli was transformed by the calcium chloride method (Maniatis *et al.*, 1982). Yeast strains were transformed using the DMSO/LiOAc/PEG procedure described by Soni *et al.* (1993).

Analysis of PHB and fatty acids. PHB content was determined by GC analysis of dichloroethane extracts of dried cell material subjected to propanolysis (Riis & Mai, 1988). Cells to be analysed were harvested, washed with deionized water and dried overnight at 94 °C. Dry cell material (30–60 mg) was then incubated at 100 °C for 2 h in a mixture of 0.5 ml dichloroethane plus 0.5 ml acidified propanol. The organic phase was extracted once with water, then used for GC analysis. GC samples were analysed using a Hewlett-Packard 5890A gas chromatograph.

Samples were run on a DB-WAX 30W capillary column and detected by a flame ionization detector, with a constant rate of temperature increase of $10^{\circ}\text{C min}^{-1}$ (60–200 $^{\circ}\text{C}$). Confirmation of the presence of the propyl ester of 3-hydroxybutyrate (propyl 3HB) was by GC/MS. Analysis was performed using a Kratos MS25 GC/mass spectrometer using the same column type and temperature profile as described for the GC analysis.

Fatty acid quantification was obtained from the same GC assay described above. Experiments showed that palmitic acid (Sigma) was easily esterified to the propyl ester under the assay conditions and was quantifiable; in addition, propyl palmitate elutes as a large, distinct peak in GC traces from prepared yeast samples. Palmitic acid residues were thus used as an indicator of overall fatty acid content of the cells. The identity of propyl palmitate in yeast preparations was confirmed using GC/MS.

¹H-NMR analysis. Extracts containing intact PHB for ¹H NMR were prepared by first breaking open the cells by grinding with glass beads in a Bead Beater (Biospec), then freeze-drying the resulting suspension and extracting the dried cell material by refluxing for 24 h in chloroform. After filtration, PHB was precipitated from the chloroform by addition of 5 vols methanol. The precipitate was recovered, dried and resuspended in deuterated chloroform. ¹H-NMR spectra were generated using a 300 MHz FT-NMR model 1280 (Nicolet Technology).

Western analysis of proteins. Yeast cells were lysed by vortexing in microfuge tubes with glass beads (Jazwinski, 1990). Lysis buffer contained 20 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 1 mM EDTA, 5% (w/w) glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 1 mM PMSF, and 1 × protease inhibitor mix (Dunn & Wobbe, 1990). Soluble extracts of *A. eutrophus* were prepared by passing cell paste several times through a French press followed by centrifugation at 10000 g. Total protein was assayed by the method of Bradford (1976) using a Bio-Rad kit with bovine IgG as a standard.

Polyclonal antibodies were raised in New Zealand White rabbits against the PHB synthase enzyme of *A. eutrophus*. A plasmid was constructed using pGEX-3X (Pharmacia) and pAet41 such that the PHB synthase gene was truncated at the *EcoRV* site and fused to a glutathione *S*-transferase gene. The glutathione *S*-transferase fusion protein was overexpressed in *E. coli*, and the fusion protein was purified by affinity chromatography using glutathione-linked agarose beads. The purified fusion protein was cleaved with factor Xa (NEB) on the column, and the cleaved fusion protein was eluted and used for immunization. Rabbits were immunized twice at an interval of 5 weeks. Serum was prepared from blood samples and used without further purification.

Crude protein extracts were separated on 7.5% SDS-PAGE gels using the method of Laemmli (1970). The proteins were electrophoretically transferred to Immobilon-P PVF membranes (Millipore). The membranes were blocked using 10% (w/v) non-fat dry milk (Bio-Rad), followed by exposure to rabbit anti-PHB synthase primary antibody. Secondary antibody and substrate–chromogen solution were provided by an Immunoblot SAP kit for rabbit primary antibody (Zymed) with dilutions as recommended by the manufacturer. Protein size was estimated by comparison with broad-range prestained SDS-PAGE standards (Bio-Rad).

Enzyme assays. Assays were performed at 30 $^{\circ}\text{C}$. Yeast protein extracts were prepared by glass bead lysis in microfuge tubes as described above.

D-3HB-CoA dehydrogenase activity was assayed by the method of Saito *et al.* (1977) with slight modifications. D-3HB-CoA was

prepared by the mixed anhydride method (Wieland & Rueff, 1953). Final assay concentrations were 33.3 mM Tris/HCl, pH 8.0, 144 μM D-3HB-CoA and 416 μM NAD⁺. The reaction was initiated by addition of enzyme. The rate of formation of NADH was measured at 340 nm with a Hewlett-Packard diode array spectrophotometer model 8452A. NADH concentrations were determined from a calibration curve. Essentially, no background formation of NADH was found when D-3HB-CoA was omitted from the complete assay mixture.

3-Ketothiolase activity was measured in the thiolysis direction by the method of Stern (1955) with slight modifications. The reaction mixture contained 67 mM Tris/HCl, pH 8.1, 53 mM MgCl₂, 134 μM CoASH and 134 μM AcAc-CoA (Sigma). The reaction was initiated by addition of enzyme. The rate of disappearance of AcAc-CoA was measured at 304 nm by comparison with a calibration curve. Note that the extinction coefficient of AcAc-CoA is very sensitive to both pH and magnesium ion concentration (Stern, 1956).

Lipid staining and flow cytometry. The lipophilic fluorescent stain Nile red was used to stain PHB (Ostle & Holt, 1982) and other lipids (Greenspan *et al.*, 1985; Cooksey *et al.*, 1987). Cells were fixed in 3.7% (w/v) formaldehyde for 2 h at room temperature, then washed and resuspended in 0.1 M potassium phosphate buffer, pH 5.8. Cells were stained in a solution of 50 ng Nile red ml⁻¹ in filtered 0.85% NaCl, then washed and resuspended in 0.1 × staining solution.

Single cell fluorescence was measured using an Ortho cytofluorograph IIs illuminated with a Coherent Innova 90-5 argon ion laser operating with a power output of 100 mW at 488 nm. Fluorescence data were collected in log and area mode through a 570 nm long pass filter. For each sample 20000 cells were analysed.

Freeze-fracture electron microscopy. Cells for freeze-fracture were grown in a bioreactor as described, well into stationary phase. Cells were harvested, washed in 0.1 M potassium phosphate buffer (pH 7) and fixed in 2% (v/v) glutaraldehyde for 2 h on ice in the same buffer, followed by washing. Cells were frozen in 25% (w/v) glycerol and freeze-cleaved at 77 K according to the method of Sleytr (1970). The preparations were then shadowed with platinum/carbon and images generated by transmission electron microscopy.

RESULTS AND DISCUSSION

Synthesis of PHB in *S. cerevisiae* carrying PHB synthase plasmid

The yeast plasmid pTL85, carrying the PHB synthase gene from *A. eutrophus* under the control of a yeast *GAL10/CYC1* hybrid promoter (Guarente *et al.*, 1982), is shown in Fig. 1. The plasmid carries the high copy number yeast 2 μm plasmid origin of replication and a *URA3* selection marker. The translation start codon is provided by the vector and is flanked by yeast DNA, a feature which is expected to improve the attainable level of heterologous expression in yeast (Mellor *et al.*, 1985; Cigan & Donahue, 1987). This translational fusion was made after constructs containing the entire structural gene plus bacterial flanking regions failed to yield detectable synthase activity in yeast.

The promoter–gene fusion was made using available restriction sites and as a result the putative protein product

Fig. 2. PHB synthesis in (a) plasmid-free host strain D603 and (b) recombinant D603(pTL85) cultivated in bioreactors with galactose as sole carbon source. Biomass concentration ($\diamond$), specific PHB content ($\blacklozenge$), galactose concentration ($\bullet$) and ethanol concentration ($\circ$) are shown.

is truncated. The PHB synthase gene is known to be insensitive to moderate truncation at its N-terminus. Schubert *et al.* (1991) reported that a truncated gene lacking the first 107 nucleotides produces fully active synthase when expressed in *E. coli*, and that a gene lacking the first 299 nucleotides produced enzyme having 7% of the full protein activity. In addition, PHB accumulation was induced in *E. coli* in our lab by a plasmid carrying a PHB operon with a glutathione *S*-transferase/synthase fusion gene lacking the first 210 nucleotides of the synthase. It was therefore expected that the truncated synthase gene in pTL85, lacking the first 140 nucleotides, would give rise to functional enzyme.

Yeast strains D603 and YPH500 were transformed with the PHB synthase plasmid pTL85 and tested for their ability to synthesize PHB. As an initial screening, cells were grown in shake flasks on SD medium and then assayed for PHB. In all media the initial sugar concentration was 20 g l⁻¹, so that sugar was not the limiting nutrient (DaSilva, 1988; Fig. 2). ODs of the cultures were monitored and cells were harvested for analysis after 15–20 h into stationary phase. This corresponds to a culture age of 47 h with glucose as carbon source, 64 h with galactose as carbon source and 93 h with galactose medium containing compactin. All flasks were grown in the same incubator-shaker, and in each case three separate flasks were inoculated from the same seed culture and PHB content determined separately for each flask. Data from shake flasks indicated that plasmid-free cells did not

contain measurable PHB, nor did plasmid-containing cells grown under non-inducing conditions (glucose as sole carbon source). In contrast, plasmid-containing cells grown under inducing conditions contained PHB in the order of 1 mg PHB (g dry wt)⁻¹. The degree to which PHB accumulated in recombinant cells seemed to have some dependence on host strain, as evidenced by the difference between PHB levels produced in strains D603(pTL85) [1.4 mg PHB (g dry wt)⁻¹] and YPH500/pTL85 [0.31 mg PHB (g dry wt)⁻¹]. The presence of 28 mg compactin l⁻¹, an enzyme inhibitor discussed further below, seemed to cause a threefold increase in the PHB level attained by D603(pTL85) in stationary phase [3.9 mg PHB (g dry wt)⁻¹]. Coefficients of variation for these measurements were typically 20%.

The results verify the functionality of the PHB synthase protein expressed from the plasmid. Evidence that PHB synthase requires a phosphopantetheine prosthetic group for catalytic activity (Gerngross *et al.*, 1994) suggests that the yeast cell is able to carry out this post-translational modification on this heterologous protein. PHB synthesis also implies that D-3HB-CoA, the substrate for PHB synthase, is somehow formed in the cells without the presence of the other PHB synthetic enzymes of *A. entrophus*, a process that does not seem to occur in other cell types. Expression of the synthase gene alone in *E. coli* produces no PHB (Peoples & Sinskey, 1989). Transgenic *Arabidopsis thaliana* plants expressing only a synthase gene similarly do not produce PHB (Poirier *et al.*, 1992). These results do not rule out the possibility that the necessary auxiliary enzymes exist in these host cells but were not expressed under the conditions used for study.

The shake flask results must be interpreted with caution, since they do not reveal whether PHB synthesis occurred during growth or, as is observed for *A. entrophus*, during non-growth conditions. These experiments were followed with batch culture in bioreactors, focussing on strain D603(pTL85) as it appeared to accumulate the greatest amounts of PHB.

Kinetics of PHB formation in yeast

The pattern of PHB accumulation in yeast over the course of batch growth was followed during controlled culture in a bioreactor. The recombinant as well as the plasmid-free host strain were grown aerobically at 30 °C and pH 4.5 in bioreactors on enriched SD medium containing initially 20 g galactose l⁻¹, lacking uracil where appropriate. This medium was nitrogen-limited, with galactose levels near 5 g l⁻¹ when the cells reached stationary phase. The cultures were sampled over the course of batch growth for PHB and protein analysis.

Representative data from bioreactor runs are shown in Fig. 2. The untransformed host strain generated a GC signal corresponding to a PHB content of 0.2–0.5 mg PHB (g dry wt)⁻¹. This level is about two orders of magnitude higher than that reported by Reusch (1989) for wild-type *S. cerevisiae* cells, and about one order of magnitude lower than that reported by Nuti & Lepidi

Fig. 3. GC/MS analysis of cell extracts, indicating the presence of PHB in recombinant yeast. Dried cell material was subjected to propanolysis and solvent extraction. Comparison of GC traces of (a) host strain D603 and (b) recombinant D603(pTL85) shows a peak (arrow) corresponding to the same elution time as the propyl 3HB standard (not shown). MS analysis of (c) a propyl 3HB reference sample and (d) the material suspected to be propyl 3HB in the recombinant cell extract confirms the identity of the GC peak in (b).

Fig. 4. Scanned image of a Western blot of proteins from yeast strains D603 and D603(pTL85). The *A. eutrophus* (A.e.) protein is included as a reference. The position of the putative truncated synthase produced by the recombinant is indicated by the arrow. All yeast proteins were loaded onto the gel at 100 µg per lane, *A. eutrophus* protein at 50 µg.

(1974). Identification of GC peaks as propyl 3HB was made using GC/MS (Fig. 3).

In recombinant D603(pTL85), PHB content was essentially equal to wild-type levels during exponential growth but a definite accumulation of PHB occurred in the stationary phase. The final PHB level in recombinant cells represents a 10-fold increase as compared to plasmid-free cells when grown on galactose as the sole carbon source. Levels of PHB at an extended cultivation time of 120 h approached 6 mg PHB (g dry wt)⁻¹ for the recombinant (data not shown). During the accumulation phase, the maximum rate of PHB synthesis was about 2.5 µg PHB (g dry wt)⁻¹ h⁻¹ or 0.03 µmol PHB monomers (g dry wt)⁻¹ h⁻¹. This rate of PHB synthesis, on a per unit non-PHB biomass basis, is about 10⁵ times less than that observed during PHB accumulation in *A. eutrophus*

(Pedrós-Alió *et al.*, 1985; Heinzle & Lafferty, 1980). Interestingly, PHB accumulation coincided with ethanol production, suggesting that the same metabolic conditions (e.g., availability of carbon and an excess of reducing equivalents) favour both processes.

Western blot identifies truncated PHB synthase

Western blots were performed to qualitatively evaluate levels of truncated synthase protein. Cell samples for protein analysis were collected from the reactor runs depicted in Fig. 2. An image of a Western blot prepared from crude cell-free extracts is shown in Fig. 4. Extracts from D603(pTL85) reveal a band near 60 kDa, the size of the putative truncated protein, that does not appear in extracts from the plasmid-free host strain. This band

appears to weaken as cells progress through the PHB accumulation phase. A possible explanation for this is that the synthase becomes associated with PHB granules and is removed from the soluble fraction during preparation of protein extracts (Nawrath *et al.*, 1994).

Since antibodies were raised against a truncated synthase, the intensity of Western blots should reflect molar protein concentrations for both full-length and truncated synthases. Densitometry on the Western blot suggests that the recombinant yeast cells contain up to 30%, on a per unit protein basis, of the molar concentration present in *A. eutrophus* cells. This seems high in light of the very low rate of PHB synthesis observed in recombinant yeast. Although a number of factors may affect the densitometry quantification, the results suggest that enzyme activities other than the synthase are limiting the rate of synthesis of PHB. Compartmentation of pathway enzymes or precursors could also affect rates of PHB synthesis.

¹H-NMR identifies PHB

Solvent extracts of yeast cells prepared as described were subjected to ¹H-NMR (Fig. 5). For strain D603, 24.7 g dry cell material containing 0.19 mg PHB (g dry wt)⁻¹ (by GC analysis) yielded 29 mg methanol-precipitated material for ¹H-NMR. The ¹H-NMR spectrum obtained from this extract (Fig. 5c) failed to show either PHB or 3HB. For the recombinant strain D603(pTL85), 14.7 g dry cell material containing 4.0 mg PHB (g dry wt)⁻¹ (by GC analysis) yielded 10 mg methanol-precipitated material. ¹H-NMR of this material (Fig. 5d) showed the characteristic methylene and methine proton resonances found in PHB (Fig. 5a). The monomer 3HB (free acid) displays a methine proton resonance with a significantly lower chemical shift (Fig. 5b), and is not apparent in extracts from either wild-type or recombinant cells. Our conclusions from ¹H-NMR data are that the recombinant yeast cells do in fact synthesize polymer, while the wild-type cells appear to contain no polymer.

The methodology used for PHB detection deserves some reflection. Most researchers have analysed PHB using techniques in which it is not the polymer but a derivatized product that is detected. Examples of this are the method of Law & Slepecky (1961) where concentrated sulfuric acid induces conversion of the polymer to crotonic acid, and the method of Riis & Mai (1988), used in this study, in which reaction with acidified propanol forms propyl 3HB. The drawback to these chemical methods is the likelihood that non-polymeric moieties such as 3HB or its CoA thioester will derivatize along with the polymer and distort the measurement.

Fortunately, it is possible to unambiguously distinguish between polymer and monomeric species using ¹H-NMR. Problems with this technique are the relatively large amount of material required as compared to chemical methods, and interference by contaminants in cell extracts. A survey of some cell types not known to accumulate PHB (Seebach *et al.*, 1994), yeast not included, demonstrated by ¹H-NMR that the polymer was in fact present.

Fig. 5. ¹H-NMR spectra for (a) PHB, (b) 3-hydroxybutyric acid, (c) chloroform-extracted, methanol-precipitated material from wild-type yeast and (d) recombinant D603(pTL85). PHB proton resonances are clearly visible in the recombinant (d). All samples were dissolved in d-chloroform. The PHB structure with numbered carbon atoms is represented at the upper left.

These authors also discussed difficulties in detecting the small amounts of PHB, with the requirement for gel chromatographic purification to obtain satisfactory ¹H-NMR spectra from some samples.

PHB in wild-type yeast was originally reported by Reusch (1989) and to our knowledge has never been confirmed by ¹H-NMR (Reusch, 1992; Seebach *et al.*, 1994). These authors have not attempted monomer detection by ¹H-NMR, though such species may be relatively insoluble in chloroform. Although we could not detect polymer in our wild-type strains, questions remain about ¹H-NMR detection limits. The wild-type ¹H-NMR sample, from which no PHB signal was detected, contained only 45 µg PHB as indicated by the GC assay. Questions also remain about the extraction method employed. Reusch (1992) suggests that native low molecular mass PHB may be complexed with various lipids or proteins, making its

Fig. 6. Freeze-fracture electron micrographs of PHB-containing yeast strain D603(pTL85). Cell material contained 6 mg PHB (g dry wt)⁻¹. Plastic deformations of PHB granules are indicated by arrows. Free-standing horn-like extrusions (a, magnified in b) and collapsed extrusions (c, magnified in d) are visible.

solubility behaviour unpredictable. We have observed a distinct loss of GC signal when wet cells are washed in acetone prior to the propanolysis assay (data not shown). However, loss of GC signal was minimal when dried cell material was extracted with acetone or chloroform. Our evidence suggests that chloroform-insoluble non-polymeric species are responsible for at least part of the GC signal in wild-type yeast cells.

Freeze-fracture electron microscopy reveals PHB granules

Freeze-fracture electron micrographs of strain D603(pTL85) (Fig. 6) displayed horn-like extrusions that are typical of those seen in freeze-fracture images of PHB-containing bacteria. These protuberances result from plastic deformation of PHB granules (Sleytr & Robards,

1977) and appear as dark objects casting light shadows which give an indication of their height. Sometimes the stretched granule collapses onto the adjacent surface. Also characteristic of plastically deformed PHB granules are 'stretch marks' found in granules at the base of such extrusions. Such artifacts have not been found in freeze-fracture electron micrographs of wild-type *S. cerevisiae*. The micrographs indicate that PHB in recombinant yeast occurs as discrete granules located within the cytoplasm, typically in clusters.

PHB synthesis in the presence of enzyme inhibitors

Postulated pathways by which yeast could synthesize the PHB precursor D-3HB-CoA include one involving the fatty acid synthesis pathway and another involving an enzyme of β -oxidation (Fig. 7). In the former case, premature release of D-3HB as a CoA thioester from the

Fig. 7. Hypothetical pathways to PHB synthesis in yeast strain D603(pTL85). See text for discussion. Intermediates in the *A. eutrophus* PHB pathway are boxed. Enzyme inhibition is indicated by dashed arrows.

fatty acid synthetase (FAS) would provide PHB precursors. In the latter case, a β -oxidation enzyme possessing D-3HB-CoA dehydrogenase activity would convert AcAc-CoA to D-3HB-CoA.

The participation of FAS is suggested by the work of Joshi & Smith (1993) in which *in vitro* enzyme systems demonstrated accumulation of D-3HB-CoA by a mutated rat FAS deficient in the dehydrase function. Such a process might occur more efficiently in yeast, where the natural product of FAS is a coenzyme A thioester, as compared to mammalian cells, where the FAS produces a free fatty acid (Wakil *et al.*, 1983). On the other hand, a β -oxidation protein with the necessary dehydrogenase activity has been found in yeast (Hiltunen *et al.*, 1992) and could catalyse formation of D-3HB-CoA. To elucidate the pathway by which yeast make D-3HB-CoA, the kinetics of PHB formation in the presence of two different specific enzyme inhibitors were observed.

Cerulenin, a suicide substrate for FAS, covalently and irreversibly bonds to the condensing enzyme domain of yeast FAS (Funabashi *et al.*, 1989), which prevents formation of the four-carbon intermediate needed for PHB synthesis. If D-3HB-CoA is formed by FAS, cerulenin would be expected to cause a marked decrease in the rate of PHB synthesis. Compactin (mevastatin) (ML-236B) (Endo *et al.*, 1976) is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the enzyme forming mevalonate from HMG-CoA in the sterol pathway. The first step in the synthesis of mevalonate from Ac-CoA is catalysed by a cytoplasmic thiolase forming AcAc-CoA. Compactin acts downstream from this reaction (Fig. 7) and might lead to accumulation of AcAc-CoA, which could be a precursor for PHB synthesis.

Evaluation of the effect of these inhibitors on PHB synthesis was done by first growing cells to early stationary phase in bioreactors to maintain pH and aeration, then splitting cultures into three shake-flasks: one containing cerulenin (1.2 mg l^{-1}), one containing compactin (8.5 mg l^{-1}) and one containing no inhibitor as a control. The cerulenin concentration is somewhat less than the 5.6 mg l^{-1} used by Duronio *et al.* (1992) to inhibit yeast growth; the concentration of compactin is similarly less than the 40 mg l^{-1} used by Basson *et al.* (1986) to retard yeast growth on plates. PHB, biomass, Nile red fluorescence and palmitate data are summarized in Fig. 8. It can be seen that neither enzyme inhibitor had an effect on PHB synthesis, while both affected total lipid content as judged by mean Nile red fluorescence levels. The effect of cerulenin was further confirmed by the drop in palmitate content of the treated cells. Compactin-treated wild-type cells greatly decreased in fluorescence even though PHB and palmitate remained constant, suggesting that this antibiotic was inhibiting sterol synthesis.

Blocking sterol synthesis downstream from AcAc-CoA could lead to accumulation of this species, but regulatory mechanisms may exist for maintaining the size of metabolite pools. Additionally, if the natural level of AcAc-CoA is close to equilibrium with Ac-CoA, then a reduction in the rate of reactions consuming AcAc-CoA would not be expected to cause much accumulation. Therefore, if D-3HB-CoA is synthesized via AcAc-CoA, then compactin may or may not be expected to affect the rate of PHB synthesis. The compactin data, showing essentially no effect on PHB synthesis, thus do not support or disprove the existence of such a pathway for D-3HB-CoA synthesis in yeast. In contrast, the cerulenin data allow one to conclude that PHB precursors are not synthesized via the fatty acid pathway, since the reaction blocked by cerulenin would be an essential step in this pathway.

D-3-HB-CoA dehydrogenase activity in yeast

The stereospecificity of the PHB synthase requires the D-enantiomer of 3HB-CoA as a substrate for polymerization (Haywood *et al.*, 1989). Yeast cells could synthesize D-3HB-CoA if they possessed AcAc-CoA reductase activity. An assay in which AcAc-CoA is the substrate and NAD(P)H is oxidized concomitantly with 3HB-CoA formation would not directly give information on the chirality of the product. One can ascertain the stereospecificity of the enzyme in question by using an optically pure 3HB-CoA as substrate to measure 3HB-CoA dehydrogenase activity. We thus proceeded to measure D-3HB-CoA dehydrogenase activity in wild-type yeast cells. Completion of the PHB pathway from Ac-CoA in a synthase-producing strain would also require AcAc-CoA thiolase activity, and this was also measured.

In vitro assays of yeast crude protein extracts (Fig. 9) indicated that D-3HB-CoA dehydrogenase activity exists in galactose-grown wild-type cells at a level of $150\text{--}200 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. D-3HB-CoA dehydrogenase activity has been reported in yeast (Hiltunen

Fig. 8. Dry weight, PHB, Nile red fluorescence and palmitate data for batch culture of (a) host strain D603 and (b) recombinant D603(pTL85). Cultures were grown in a bioreactor to early stationary phase then transferred to shake flasks for treatment with enzyme inhibitors. The time point for splitting cultures is indicated by arrows. Flask treatments were: ●, cerulenin (1.2 mg l⁻¹); □, compactin (8.5 mg l⁻¹); ○, no treatment. Flow cytometric fluorescence data were collected in log mode; each plotted value is the mean channel number for 20000 cells.

et al., 1992) but not quantified. Haywood *et al.* (1988b) reported 230 nmol min⁻¹ (mg protein)⁻¹ in nitrogen-limited cells of *A. entrophus* (oxidation of D-3HB-CoA); caution is warranted in comparison since our assay conditions are slightly different. It is also important to recognize that our assays provide no information about compartmentation of enzyme activities within the yeast cell.

Assays also showed AcAc-CoA thiolase activity in wild-type yeast at a level of 10–20 nmol min⁻¹ (mg protein)⁻¹. Haywood *et al.* (1988a) reported 200 nmol min⁻¹ (mg protein)⁻¹ in nitrogen-limited cells of *A. entrophus* (thiolysis reaction). References to thiolase activity in yeast under various growth conditions can be found. Trocha & Sprinson (1976) report 7.7 nmol min⁻¹ (mg protein)⁻¹ in anaerobically grown cells aerated for 2 h in nitrogen-free glucose medium. Thiolase activity of 140 nmol min⁻¹ (mg protein)⁻¹ in exponentially growing cells (YPD medium) was measured by Servouse & Karst (1986).

Yeast grown aerobically on galactose showed thiolase activity of 10–100 nmol min⁻¹ (mg protein)⁻¹ (Kornblatt & Rudney, 1971), depending on the growth state of the culture. In the peroxisomal fraction of oleic acid-grown cells, 1.49 nmol min⁻¹ (mg protein)⁻¹ was found (McCammon *et al.*, 1990). Igual *et al.* (1992) found thiolase activity in oleate-grown cells to peak at about 16 nmol min⁻¹ (mg protein)⁻¹ in the early stationary phase, with levels as low as 2 nmol min⁻¹ (mg protein)⁻¹ during exponential growth. Chemostat cultures grown on oleic acid possessed 15 nmol min⁻¹ (mg protein)⁻¹ (Evers *et al.*, 1991).

Having shown the necessary enzyme activities in wild-type yeast, we postulate a pathway forming D-3HB-CoA from Ac-CoA via an AcAc-CoA intermediate, as occurs in *A. entrophus*. In strain D603(pTL85), PHB synthase is expected to be expressed in the cytoplasm. This suggests the participation in PHB synthesis of the yeast Erg10 protein (Fig. 7), a cytoplasmic thiolase involved in

Fig. 9. Enzyme activity in wild-type yeast. Cell growth (◇) and GC assay for (a) PHB (◆), (b) D-3HB-CoA dehydrogenase activity and (c) thiolase activity are shown.

mevalonate synthesis (Servouse *et al.*, 1984). Formation of D-3HB-CoA from AcAc-CoA might occur through the action of the peroxisomal Fox2 protein, which has D-3HB-CoA dehydrogenase activity and participates in β -oxidation (Hiltunen *et al.*, 1992). Interestingly, β -oxidation in yeast was previously thought to occur through L-3-hydroxyacyl-CoA intermediates. The participation of Fox2p in PHB synthesis would be unexpected for a couple of reasons. Firstly, the β -oxidation enzymes are induced by oleic acid, a substance not provided in our growth media. Secondly, β -oxidation proteins are expected to be localized in peroxisomes, away from the presumed cytoplasmic site of PHB synthesis in D603(pTL85). However, peroxisomal proteins are synthesized on free polyribosomes in the cytoplasm and imported post-translationally and may be temporarily active in the cytoplasm. If this is the case, then the enzyme activity in crude cell extracts may not reflect the actual activity available for cytoplasmic PHB synthesis. Another scenario could be the synthesis of D-3HB-CoA in peroxisomes

followed by transport of 3HB moieties to the cytoplasm. The question of whether Fox2p is involved in PHB synthesis could be resolved by study of *fox2* mutants.

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