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Poly(3-hydroxyalkanoate) polymerase synthesis and in vitro activity in recombinant *Escherichia coli* and *Pseudomonas putida*

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Abstract We tested the synthesis and in vitro activity of the poly(3-hydroxyalkanoate) (PHA) polymerase 1 from *Pseudomonas putida* GPo1 in both *P. putida* GPp104 and *Escherichia coli* JMU193. The polymerase encoding gene *phaC1* was expressed using the inducible P*alkB* promoter. It was found that the production of polymerase could be modulated over a wide range of protein levels by varying inducer concentrations. The optimal inducer dicyclopropylketone concentrations for PHA production were at 0.03% (v/v) for *P. putida* and 0.005% (v/v) for *E. coli*. Under these concentrations the maximal polymerase level synthesized in the *E. coli* host (6% of total protein) was about three- to fourfold less than that in *P. putida* (20%), whereas the maximal level of PHA synthesized in the *E. coli* host (8% of total cell dry weight) was about fourfold less than that in *P. putida* (30%). In *P. putida*, the highest specific activity of polymerase was found in the mid-exponential growth phase with a maximum of 40 U/g polymerase, whereas in *E. coli*, the maximal specific polymerase activity was found in the early stationary growth phase (2 U/g polymerase). Our results suggest that optimal functioning of the PHA polymerase requires factors or a molecular environment that is available in *P. putida* but not in *E. coli*.

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Introduction

Poly(3-hydroxyalkanoates) (PHAs) are intracellular storage polyesters that are produced by many bacteria (Madison and Huisman 1999; Steinbuchel 2003). The best known PHA materials, for which the genetics and enzymology have been studied in detail and which have been produced industrially, are short-chain-length PHAs containing poly(3-hydroxybutyric acid) (PHB) and/or poly(3-hydroxyvaleric acid) (PHV) (Sudesh et al. 2000; Reddy et al. 2003). Most other PHAs are referred to as medium chain length, or mcl-PHAs, because the monomers are generally 3-hydroxyalkanoic acids with six or more carbon atoms (Steinbüchel and Valentin 1995). Mcl-PHAs have attracted considerable attention due to their potential applications in medicine and industry and as sources of chiral monomers (Ueda and Tabata 2003; Hrabak 1992). Therefore, research is being performed to gain insight into the physiology and biochemistry of mcl-PHA production to reduce production costs. One of the approaches is the use of recombinant *Escherichia coli* because this organism is well studied and utilizes a wide range of carbon sources. Furthermore, easy and cheap downstream processing techniques are available for *E. coli* (Fidler and Dennis 1992).

Several enzymes are involved in the synthesis of PHAs, with PHA polymerases as the key enzymes. These incorporate 3-hydroxyacyl moieties into nascent PHA chains, using coenzyme A (CoA)-linked precursors (Huijberts et al. 1995; Kraak et al. 1997a). Such CoA substrates may be synthesized by a variety of pathways (for a recent review, see Aldor and Keasling 2003). When fatty acids are used as sole carbon source, the polymerase precursor 3-hydroxyacyl-CoA is probably derived from the fatty acid β-oxidation pathway (Huijberts et al. 1995; Qi et al. 1998; Ren et al. 2000a). Mcl-PHA has been successfully produced in *E. coli* by blocking or inhibiting the β-oxidation pathway

(Langenbach et al. 1997; Qi et al. 1998; Ren et al. 2000a,b). These studies showed that only one enzyme, namely PHA polymerase, is essential for synthesis of PHAs in *E. coli*.

There are two PHA polymerases that are encoded by *phaC1* and *phaC2* in *P. putida* (Huisman et al. 1991). Both enzymes can independently lead to PHA accumulation in *E. coli* (Langenbach et al. 1997; Ren et al. 2000b). Unlike PHB polymerase (also called PHB synthase), which has been purified and studied in vitro in detail (Gerngross et al. 1993, 1994; Gerngross and Martin 1995; Rehm 2003), much remains to be learned about mcl-PHA polymerases, such as the relation between polymerase levels, polymerase activity and the final PHA level. Kraak et al. (1997b) have reported polymerase 1 levels in *P. putida* strains. However, the low amounts of polymerase (0.06–0.07% of total protein) make it difficult to study the effect of polymerase levels on polymerase activity and PHA contents in detail. Furthermore, no data have been reported for PHA polymerase levels or activities in *E. coli*.

In this paper, a fine tuned expression system was used to produce PHA polymerase in both *P. putida* and *E. coli*. Subsequently, the synthesis of PHA polymerase in relation to its activity and PHA production was compared for both *P. putida* and *E. coli*.

Materials and methods

Bacterial strains and plasmids Strains *P. putida* GPp104 (Huisman et al. 1991) and *E. coli* JMU193 (Rhie and Dennis 1995) were used in this study. Plasmids pGEc74 (Eggink et al. 1987) or pAlkSCm (Panke 1999), which contains the *alkS* regulatory gene, and pET702 (Fig. 1; Ren et al. 2000c), which contains *phaC1* expressed from the P*alkB* promoter, were used for production of PHA polymerase. pET702 has a sequence coding for 12 carboxy-terminal amino acids of the vesicular stomatitis virus glycoprotein (VSV G), to which monoclonal antibodies are available.

Recombinant DNA techniques Isolation and analysis of plasmid DNA were carried out according to Sambrook et al. (1989). *E. coli* competent cells were transformed according to standard procedures (Sambrook et al. 1989). Plasmids were transferred from *E. coli* donor strains to *P. putida* recipient strains by three parental mating as described by Ditta et al. (1980).

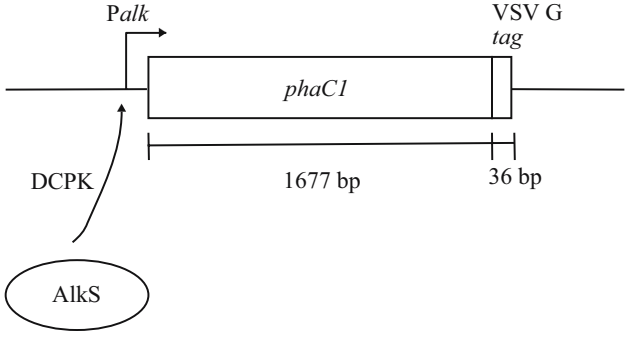

Fig. 1 *phaC1* expression from plasmids pET702 (not to scale). The expression of *phaC1* from the P*alkB* promoter is positively regulated by the AlkS polypeptide in the presence of an inducer such as DCPK

Media, growth conditions and cell disruption P. putida was precultured in complex Luria-Bertani (LB) medium at 30°C until the end of the exponential growth phase and transferred with 1:100 dilution to E2 minimal medium (Huisman et al. 1989) containing 15 mM octanoate as carbon source. *E. coli* was cultured at 37°C under the same conditions as *P. putida* except that 0.5% glycerol and 1 mM octanoate were used as substrates. If necessary, antibiotics were added: tetracycline, 12.5 μg/ml; streptomycin, 100 μg/ml. Cell growth was monitored by measuring optical density at 450 nm (OD_{450}) (Witholt 1972).

To induce the P*alkB* promoter (Fig. 1), pGEc74 or pAlkSCm were cotransformed with pET702, and dicyclopropylketone (DCPK) was added to the indicated concentrations in the early exponential growth phase.

Recombinant *P. putida* or *E. coli* cells were harvested and washed once with Tris–HCl (50 mM, pH 8). The pellets were resuspended in the same buffer to OD_{450} 40–50. Cells were disrupted by three passages through a precooled French pressure cell at 11,000 psi and 4°C.

Protein analysis and determination Proteins were separated by gel electrophoresis according to Laemmli (1970), and stained with Coomassie brilliant blue R-250. The amount of PHA polymerase relative to total protein was estimated by densitometric scanning (Molecular Dynamics). Total protein was assayed with a Lowry-based method (Biorad DC assay) in the presence of SDS with bovine serum albumin as standard. Immunoblotting of polymerase using VSV G antibodies was carried out as previously described (Staijen et al. 1997).

Determination of PHA To determine the PHA content and composition, samples were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid as previously described (Lageveen et al. 1988). The methanolyzed PHA monomers were analyzed using a 25-m ZB-1 capillary column (Brechbühler AG, Switzerland) on a gas chromatograph (Fisons, USA). Splitless injection, an attenuation of 1 and a range of 0 were used to reach maximum sensitivity.

Synthesis and purification of polymerase substrate 3-hydroxyoctanoyl-CoA Coenzyme A was coupled to *R/S*-3-hydroxyoctanoic acid (Sigma) using acyl-CoA synthetase (Sigma) as described previously (Gerhardt 1989). The product *R/S*-3-hydroxyoctanoyl-CoA was purified by using a preparative C8-spherisorb-RP column (Bischoff, Germany) together with a preparative HPLC system (Labomatic liquid chromatography system, Allschwil, Switzerland) (de Roo et al. 2000). After freeze drying the collected sample under a partial vacuum, the substrate was dissolved in 50 mM Tris–HCl (pH 8). A standard curve was made with 1 to 10 mM octanoyl-CoA (Sigma) to quantify the purified substrate.

For this purpose, an analytical column (RP ODS-2 Hypersil, Hewlett-Packard) was used with conditions applied as described previously (Kraak et al. 1997a). PHA polymerase activity in crude cell extract was measured by following the depletion of 3-hydroxyoctanoyl-CoA using HPLC as described previously (Kraak et al. 1997a). Non-induced cells were used as controls. One unit is defined as 1 μmol 3-hydroxyoctanoyl-CoA depletion per minute.

Results

Selection of expression systems for production of PHA polymerase in both P. putida and E. coli To investigate PHA polymerase 1 (PhaC1) synthesis and PHA production in *P. putida* and *E. coli*, we have brought the polymerase encoding gene *phaC1* under the control of P*lac* and P*tac* promoters (Ren et al. 2005). These constructs, however, resulted in very low levels of polymerase, less than 0.05% of total cell protein (data not shown). Previously we reported that expression of *phaC1* from the P*alkB* promoter (Fig. 1) led to 20–25% PhaC1 of total protein (w/w) (Fig. 2) and 30% PHA of total cell dry weight (cdw; w/w) in *P. putida* (Ren et al. 2000c). Thus, in this study the P*alkB–phaC1* expression system was further tested in *E. coli*. Upon induction with DCPK, the *E. coli* recombinant efficiently synthesized PHA polymerase (Fig. 2), and contained 3.6% PHA of cdw.

Influence of phaC1 induction on PHA production in both E. coli and P. putida Since full induction of a promoter does not always result in highest activity of the gene product (Hoffmann et al. 1987; Giza and Huang 1989; Elvin et al. 1990; Diederich et al. 1994), we assessed the susceptibility to modulation of the P*alkB–phaC1* constructs by measuring the amount of PHA polymerase formed and the PHA content after induction with different concentrations of DCPK.

In *P. putida* GPp104[pET702, pGEc74] (Fig. 3a), the PHA polymerase increased rapidly from 0.2 to 9% of total protein when the DCPK concentration increased from 0.005

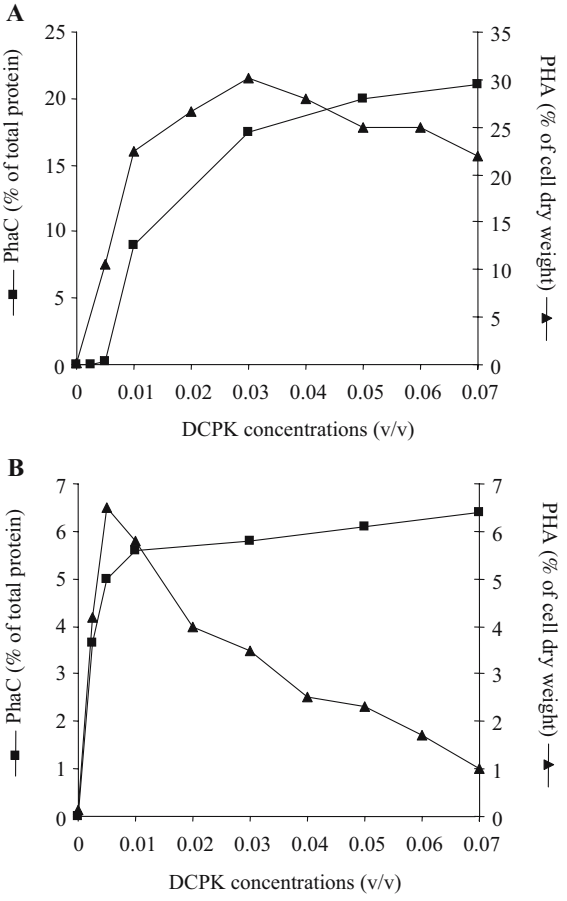

Fig. 3 Effect of DCPK concentrations on polymerase levels and PHA content in *P. putida* (**a**) and *E. coli* (**b**). *P. putida* GPp104 [pET702, pGEc74] and *E. coli* JMU193[pET702, pAlkSCm] were grown on E2 minimal medium. When cultures reached the early exponential growth phase, DCPK was added to the indicated concentrations. Cells were harvested after 10 h for polymerase measurement and after 36 h for analyzing PHA content. Data are the mean of duplicates from one experiment. The standard deviation was about 7%. Similar patterns were observed in at least three independent experiments

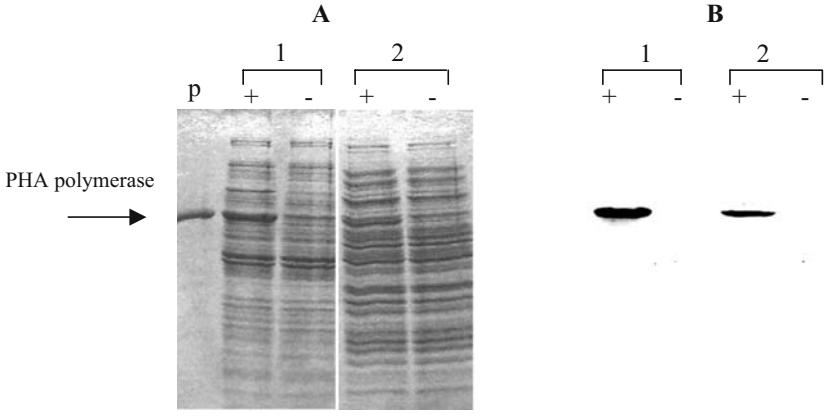

Fig. 2 Overproduction of tagged polymerase in *P. putida* and *E. coli* recombinants analyzed using VSV G antibodies. *P. putida* and *E. coli* recombinants were grown in minimal medium E2 as described in "Materials and methods". In early exponential growth phase, DCPK was added to 0.03% (v/v) and incubation continued for 4 h. Production of PHA polymerase was shown by SDS–PAGE (**a**) and Western blotting (**b**). +, induced; −, uninduced; *P*, partially purified PHA polymerase; *Lane 1*, *P. putida* GPp104[pET702, pGEc74]; *lane 2*, *E. coli* JMU193[pET702, pAlkSCm]

to 0.01%. At higher DCPK concentrations, from 0.01 to 0.07%, the polymerase level increased less dramatically, from 9 to 20% of total protein. Polymerase was not detectable (≤0.1% of total protein) without induction. The effect of DCPK concentrations on the PHA content followed a different pattern from that of the polymerase. The

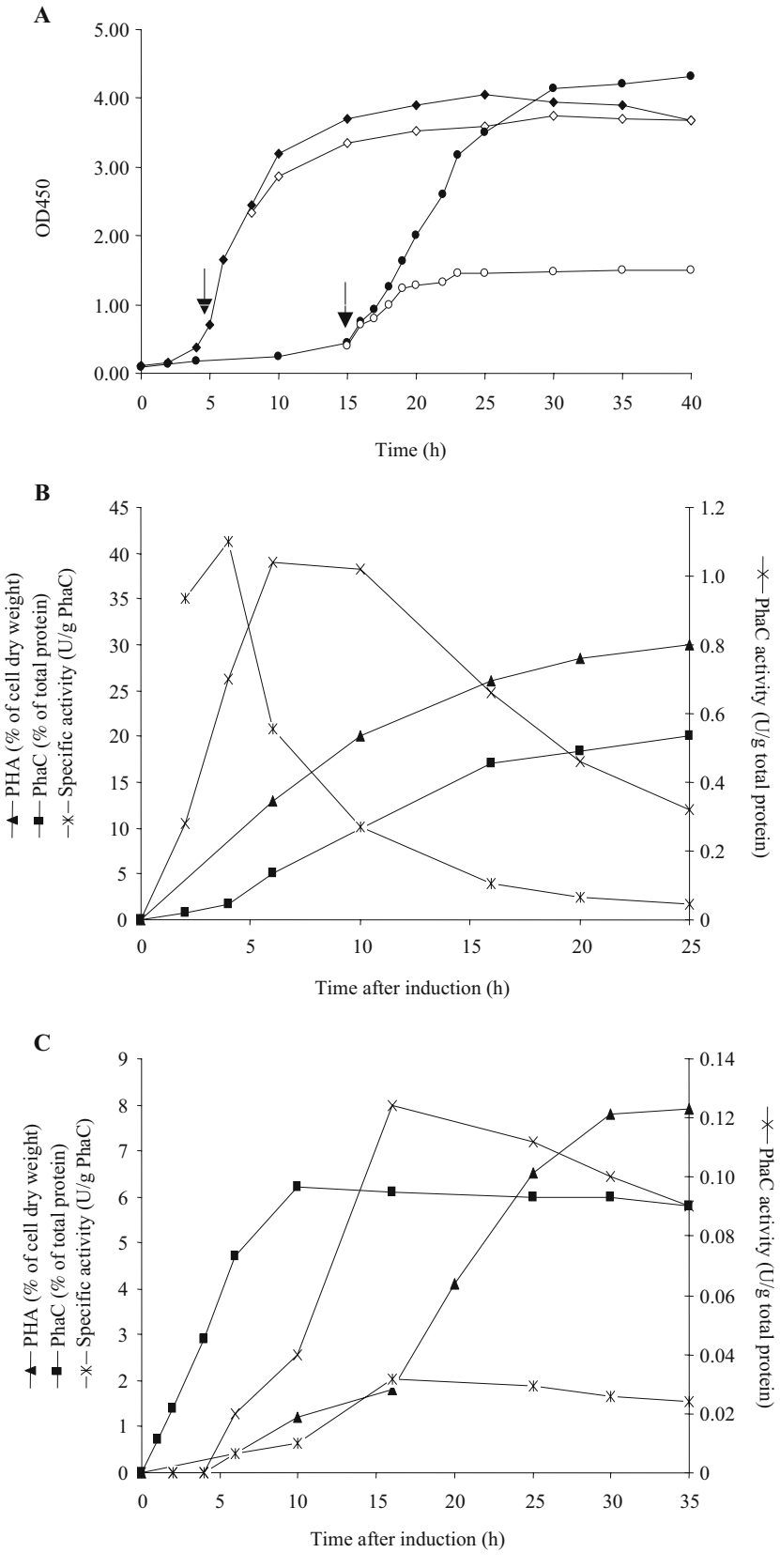

Fig. 4 Growth, polymerase synthesis, in vitro polymerase activity and PHA synthesis in *P. putida* and *E. coli*. *P. putida* GPp104[pET702, pGEc74] and *E. coli* JMU193[pET702, pAlkSCm] were cultivated in E2 minimal medium. In early exponential growth phase, cells were induced with 0.03% DCPK (for *P. putida*) and 0.005% DCPK (for *E. coli*). **a** Cell growth measured by optical density. *Open* and *closed triangles*, uninduced and induced JMU193[pET702, pAlkSCm], respectively; *Open* and *closed circles*, uninduced and induced GPp104[pET702, pGEc74], respectively; *Arrows*, DCPK was added at this time point. **b**, **c** Polymerase levels, in vitro activities of polymerase, specific activities of polymerase and PHA levels after induction in *P. putida* (**b**) and *E. coli* (**c**). Data are the mean of duplicates from one experiment. The standard deviation was about 7%. Similar patterns were observed in at least three independent experiments

PHA content increased from less than 1% PHA without induction to about 30% as the DCPK concentration increased from 0 to 0.03% (Fig. 3a). A further increase of the DCPK concentration resulted in a modest decrease of the PHA content, although the amount of the PHA polymerase continued to increase (Fig. 3a).

In *E. coli* JMU193[pET702, pAlkSCm] (Fig. 3b), production of polymerase was more sensitive to the DCPK concentration than that in the *P. putida* recombinant. PhaC1 was induced very efficiently and near maximum polymerase (5.7% of total protein) was produced at only 0.01% DCPK. Higher concentrations of DCPK led to only slight increase of PhaC1 to about 6.4% of total protein (Fig. 3b). Similarly, PHA production in *E. coli* was also more sensitive to DCPK than that in *P. putida*: cells produced up to 6.5% PHA (w/w) in response to 0.005% DCPK induction; further increase of DCPK concentration led to a significant decrease of PHA. At DCPK concentration of 0.07%, only 1% PHA (w/w) was detected. Experiments to test the toxicity of DCPK induction to *E. coli* cells showed that DCPK concentrations below 0.07% (v/v) did not influence the final cell density.

PHA polymerase synthesis in *P. putida* and *E. coli*

Under optimal induction conditions for PHA accumulation (0.03% DCPK for *P. putida* and 0.005% DCPK for *E. coli*), the polymerase production was followed for 40 h in *P. putida* as well as in *E. coli*.

In both *P. putida* and *E. coli* recombinants, especially in *P. putida* GPp104[pET702, pGEc74], induction resulted in much higher cell optical density (OD_{450} is about 4.3) than without induction (OD_{450} is about 1.4) (Fig. 4a). The obtained higher optical density of induced samples was probably caused by PHA accumulation. This phenomenon has been observed repeatedly for *P. putida* and other organisms (Durner 1998).

Polymerase levels were determined by following the appearance of the 62-kDa PhaC1 band on SDS–polyacrylamide gels and by Western blotting (Fig. 2). Before induction, the 62-kDa band could be observed neither in *P. putida* nor in *E. coli* samples, not even after prolonged exposure times of Western blots. In *P. putida* GPp104[pET702, pGEc74], polymerase synthesis slightly lagged after DCPK induction (Fig. 4b), whereas in *E. coli* JMU193[pET702, pAlkSCm], a rapid increase of PhaC1 was observed immediately after the addition of DCPK (Fig. 4c). The polymerase level rose to a maximum towards the end of the exponential growth phase in both strains: about 20% of total protein in *P. putida* recombinant (Fig. 4b), and about 6% of total protein in *E. coli* recombinant (Fig. 4c). The rate at which the polymerase content increased was close to 0.4% per hour for *P. putida* before the mid-exponential growth phase and 1.2% per hour afterwards. The rate was 0.62% per hour for *E. coli*.

In vitro PHA polymerase activities in *P. putida* and *E. coli*

We also investigated how synthesis of PhaC1 related to the polymerase activity in both strains. Before induction, PHA polymerase activity could not be detected (<0.1 U/g total protein) in either of the recombinants. Upon induction with DCPK, the specific in vitro polymerase activity in *P. putida* increased rapidly to a maximum of approximately 40 U/g polymerase in the mid-exponential growth phase (Fig. 4b). Afterwards, PhaC1 activity decreased dramatically (Fig. 4b). In *E. coli*, PhaC1 specific activity was detected in the late exponential growth phase after a lag phase (Fig. 4c). It reached a maximum of 2 U/g polymerase at the early stationary growth phase (Fig. 4c). Unlike the case in *P. putida*, PhaC1 activity in *E. coli* decreased only slightly as the growth reached the late stationary phase. The specific PhaC1 activity in *E. coli* is about 20 times lower than that seen in *P. putida*. Non-induced control cultures showed no polymerase activity, as expected.

PHA synthesis in *P. putida* and *E. coli*

The kinetics of PHA production in *P. putida* and *E. coli* upon induction with DCPK was followed for 40 h as shown in Fig. 4. Before induction, PHA could not be detected in either of these recombinants. After DCPK induction, *P. putida* GPp104[pET702, pGEc74] immediately started to accumulate PHA exponentially until the late exponential growth phase to an amount of 25% of total cdw (Fig. 4b). Then, PHA increased only slightly to 30% until the mid-stationary growth phase (Fig. 4b). In contrast, in *E. coli* JMU193[pET702, pAlkSCm] there was a lag phase for PHA accumulation after DCPK induction (Fig. 4c). The PHA production started in the late exponential growth phase and reached a maximum of about 8% of cdw at the late stationary growth phase (Fig. 4c).

Discussion

Effect of DCPK concentrations on polymerase levels and PHA contents Tight control of expression helps to avoid deleterious effects of toxic proteins due to leaky gene expression under non-inducing conditions. The P*alkB* promoter can be used for effective control of *phaC1* expression and PHA accumulation in *P. putida* GPp104 and *E. coli* JMU193 recombinants (Fig. 3).

PHA production in recombinant *E. coli* was more sensitive to DCPK concentration than that in recombinant *P. putida* (Fig. 3b). A possible explanation for this is that sufficient active PHA polymerase to process the available polymerase precursors is already produced at a DCPK concentration of 0.005% in *E. coli*. Below this level, active polymerase is probably limited, thus reducing the total amount of PHA formed. When the DCPK concentration was higher than 0.005%, more polymerase was produced,

but less PHA (Fig. 3b). Apparently, overproduction of PhaC1 in *E. coli* negatively influences its activity, perhaps by initiating incorrect protein folding and inclusion body formation, or by indirectly influencing other cell processes.

Polymerase synthesis and activity in P. putida GPp104 and E. coli JMU193 recombinants Heterologous proteins are routinely produced in *E. coli* (Hockney 1994; Makrides 1996), and here we show that it is possible to produce PHA polymerase in large amounts in *E. coli* hosts. However, absolute polymerase levels were three- to fourfold higher in *P. putida* than in *E. coli*. It is known that specific foreign genes are expressed differently by various bacterial strains, and this clearly applies also for proteins under control of the P*alkB* promoter (Nieboer 1996). Staijen et al. (1997) reported that recombinant *E. coli* W3110 was able to produce AlkB, a *P. putida* monooxygenase, under P*alkB* promoter control to 10–15% of the total protein, whereas the native *P. putida* hosts produced only 1.5–2% AlkB relative to the total protein.

In *P. putida*, the highest specific in vitro polymerase activity was found at the early exponential growth phase. It started to decrease although the polymerase levels were not decreased. The following mechanisms for the loss of polymerase activity in the stationary phase could be distinguished: (1) protein instability, (2) protein inactivation, (3) an insufficient supply of energy and reducing equivalents.

The specific activity of the system with respect to PHA polymerase in *E. coli* was observed to be 20 times lower than that in *P. putida* (Fig. 4), which indicates that there is a general systematic difference between *E. coli* and *P. putida* regarding the processing and/or assembly of a functional polymerization system or the functioning of this system. Similar results have been found for the production of PHB by *E. coli phb*$^+$ recombinants (Rhie and Dennis 1995). Possible reasons for this include subtle differences in the polymerase structure, perhaps as the result of protein folding in a slightly different environment in *E. coli*, compared to *P. putida*. Another possibility is that the absence of phasins normally located on the surface of PHA granules in *P. putida* lowers the activity of PhaC1 in *E. coli*. It was reported that PHA phasins play an important role in PHA granule structure and the enzyme activity of PHA polymerases (Potter et al. 2002; Han et al. 2004). Because *E. coli* does not have these phasins, co-expression of the phasin genes with *phaC* in *E. coli* could be a next promising step to study the differences of *phaC* expression and PHA production in *Pseudomonas* and *E. coli*.

PHA production in E. coli recombinants E. coli pha$^+$ recombinants can be as active with respect to PHA accumulation as the wild-type host *P. putida*, based on the produced polymerase. However, the cloned enzyme system does not function as well in *E. coli* as it does in *P. putida*. In this study, compared with 30% PHA found in recombinant *P. putida*, only 8% PHA was achieved in *E. coli* recombinants through application of the P*alkB–phaC1* system. As the in vitro assays indicate that the PhaC1 activity is clearly lower in *E. coli*, the lower polymerase activity is one of the reasons for the lower PHA production. However, extrinsic factors such as the availability of substrates and cofactors cannot be completely ruled out. For example, the supply of PHA precursors might be rate limiting in *E. coli*, thereby limiting PHA production, or a necessary cofactor (or cofactors) might not be supplied sufficiently by *E. coli* host strains. Indeed, we have shown that substrate channeling from of the fatty acid degradation cycle resulted in additional PHA production (Ren et al. 2000a,b). To further investigate the differences of PHA production in *Pseudomonas* and *E. coli* the extrinsic factor(s) should be studied in more detail by generating or using other *E. coli* mutants that affect the availability of possible PHA precursors.

The results presented in this paper clearly show that the PHA polymerase can be synthesized effectively in *E. coli* recombinants. These recombinants can be as active as the wild-type *P. putida* with respect to PHA synthesis, but the specific activity of the polymerase is lower than in *P. putida*. Our results indicate that there are additional native host factors (components or processes) that are vital for optimal PHA production and polymerase function and that these are not or only partially present in *E. coli* hosts. Future characterization of these factors should help us to ultimately understand the requirements for the correct assembly of a fully functional polymerization system in heterologous hosts.

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