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Expression of PHA polymerase genes of *Pseudomonas putida* in *Escherichia coli* and its effect on PHA formation

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Abstract

Poly-3-hydroxyalkanoates (PHAs) are synthesized by many bacteria as intracellular storage material. The final step in PHA biosynthesis is catalyzed by two PHA polymerases (*phaC*) in *Pseudomonas putida*. The expression of these two *phaC* genes (*phaC1* and *phaC2*) was studied in *Escherichia coli*, either under control of the native promoter or under control of an external promoter. It was found that the two *phaC* genes are not expressed in *E. coli* without an external promoter. During heterologous expression of *phaC* from P*lac* on a high copy number plasmid, a rapid reduction of the number of colony forming units was observed, especially for *phaC2*. It appears that the plasmid instability was partially caused by high-level production of PHA polymerase. Subsequently, tightly regulated *phaC2* expression systems on a low copy number vector were applied in *E. coli*. This resulted in PHA yields of over 20% of total cell dry weight, which was 2 fold higher than that obtained from the system where *phaC2* is present on a high copy number vector. In addition, the PHA monomer composition differed when different gene expression systems or different *phaC* genes were applied.

Introduction

Medium chain length poly-3-hydroxyalkanoates (mcl-PHAs) accumulated by various bacteria are of increasing industrial interest because of their broad range of potential applications such as biodegradable plastics (Hänggi 1995; Page 1995), crosslinked biodegradable rubbers (de Koning and Witholt 1996), materials in medicine and pharmacy industries (Williams et al. 1999), and sources of chiral monomers (Hrabak 1992). These biodegradable polymers can be produced from renewable substrates and therefore have the potential to replace chemically synthesized polymers, provided that the physiology, genetics, and biochemistry of the PHA-producing organisms are better understood.

Mcl-PHAs were first identified in *Pseudomonas putida* Gpo1 (earlier known as *Pseudomonas oleovorans*) grown on *n*-octane and other alkanes (de Smet et al. 1983; van Beilen et al. 2001). It has been reported that the *pha* locus from *P. putida* GPo1 encodes two PHA polymerases and a depolymerase (Figure 1) (Huisman et al. 1991). The substrate specificities of these enzymes differ slightly (Huisman et al. 1992). The two PHA polymerases are 53% identical. A homologous operon from *P. aeruginosa*

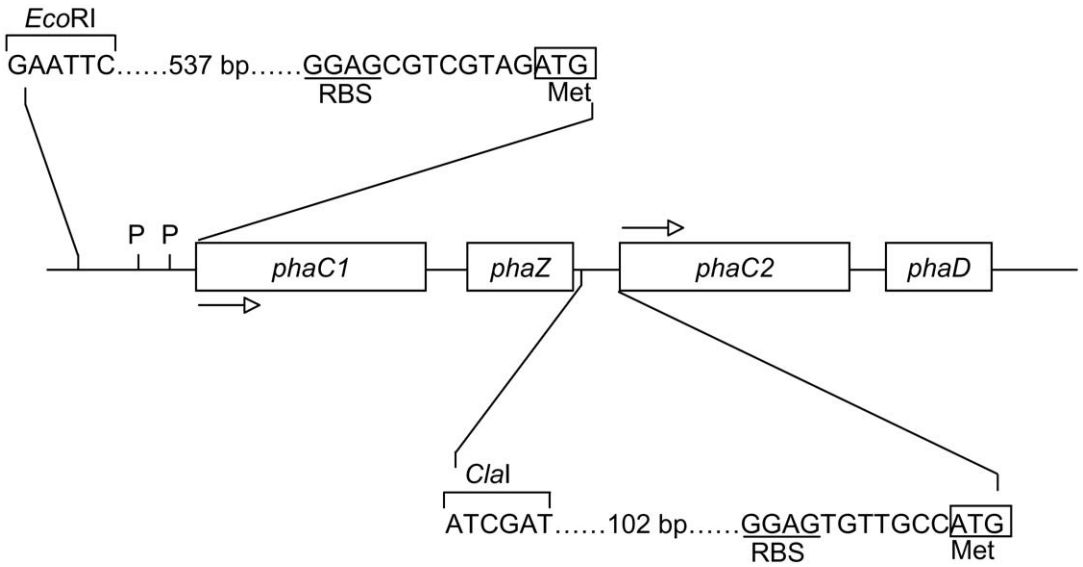


Figure 1. Organization of the *pha* genes. The scheme (not to scale) summarizes the organisation of the *pha* locus of *P. putida* Gpo1 containing at least four open reading frames (ORFs) (Huisman et al. 1991). Two polymerases and one depolymerase are encoded by *phaC1*, *phaC2* and *phaZ* genes, respectively. The *phaD* gene may encode a granule associated protein (van der Leij and Witholt 1995). Possible promoters are indicated with P. The transcriptional directions are indicated by arrows. The upper and lower parts of the Figure represent expansion of the upstream regions of *phaC1* and *phaC2*. The square boxes indicate the start codon. The ribosome binding site (RBS) is underlined. bp, base pair.

has also been identified (Timm and Steinbüchel 1992). The corresponding PHA polymerases from *P. putida* and *P. aeruginosa* show 69-80% identity at amino acid level (Huisman et al. 1991; Timm and Steinbüchel 1992).

Biosynthesis of these polymers in host organisms that do not naturally produce PHA allows modification of biosynthetic enzymes. This in turn allows increases in the quantity and quality of the products (Kidwell et al. 1995). In addition, introduction of specific genes into an organism having a suitably modified PHA synthetic pathway may allow extension and regulation of the range of compounds that is produced. *Escherichia coli* is one of these useful hosts. Indeed, many peptides and proteins of pharmaceutical value have been successfully expressed in recombinant *E. coli*. Mcl-PHA has also been successfully produced in *E. coli* by using *P. aeruginosa phaC* genes (Langenbach et al. 1997; Qi et al. 1997) and *P. putida* GPo1 *phaC* genes (Ren 1997; Ren et al. 2000a; Ren et al. 2000b) expressed from the P*lac* promoter. However, there are no reports on the stability and sustainability of heterologous *phaC* expression systems or high-copy-number plasmids in recombinant *E. coli*, even though large-scale PHA production requires stable and constant expression of *phaC* genes.

In this study, we compared expression of the two *phaC* genes from *P. putida* in *E. coli* recombinants, and investigated the stability of different *phaC* expression systems. Our results demonstrated that the two *phaC* genes cannot be expressed without an external promoter in *E. coli*. Stable and regulated expression of *phaC* genes resulted in the formation of about 20% PHA of total cell dry weight during batch cultivation. Slight differences in the composition of PHA produced with either PhaC1 or PhaC2 were observed. These stable, regulated systems for *phaC* gene expressions can serve as a first step toward establishing PHA production in recombinant strains.

Materials and methods

Strains and plasmids. *E. coli fadRfadB* strain JMU193 (Rhie and Dennis 1995) and *P. putida* GPp104 (Huisman et al. 1991) were used throughout. Plasmids pGEc407 (Huisman et al. 1991), pGEc404 (Huisman et al. 1991), pJRD215 (Davison et al. 1987), pGEM-7(+) (Promega), pMMB24 (Bagdasarian et al. 1983), pVLT33/35 (de Lorenzo et al. 1993), pCNB5 (de Lorenzo et al. 1993), pBCKS (Promega), and pUC18/19 (Yanisch-Perron et al. 1985) were used to construct the plasmids listed in Figure 2. Plasmids

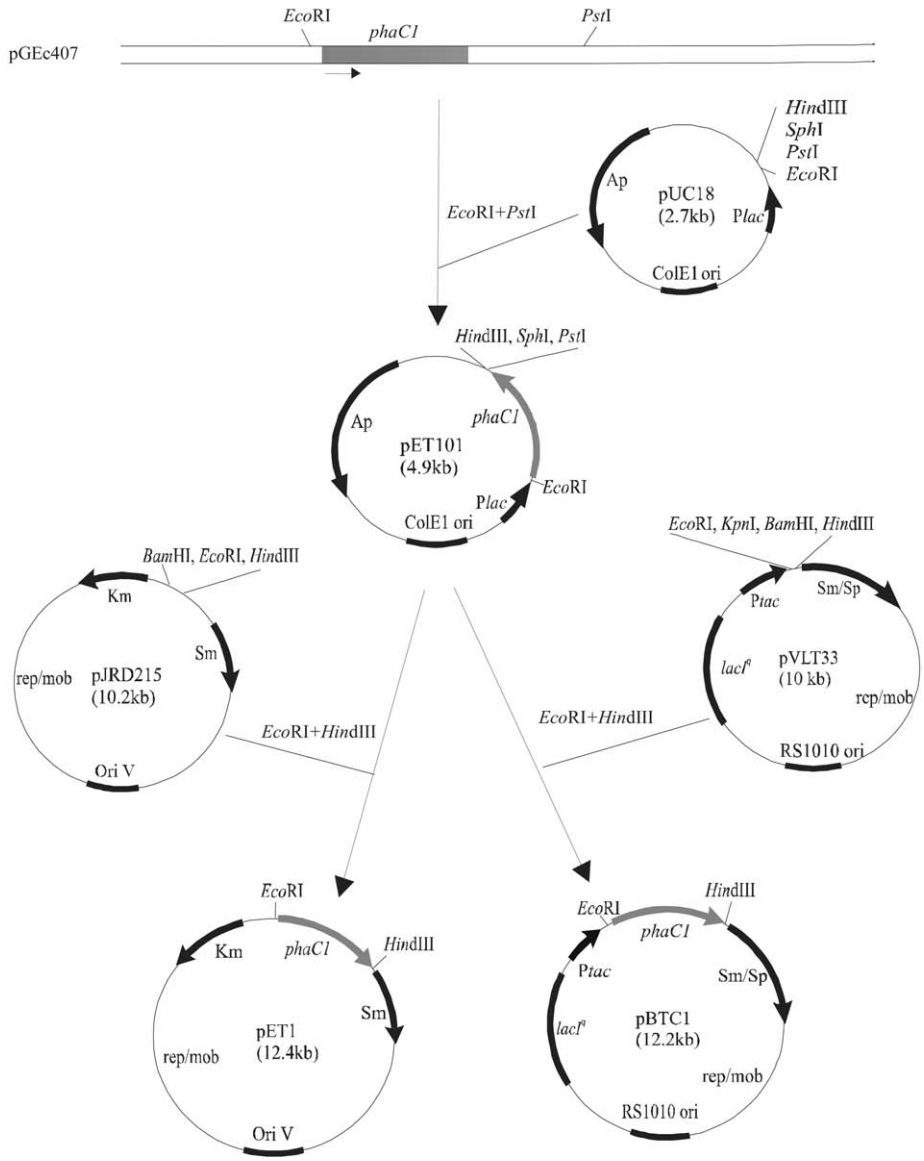


Figure 2. Schematic representation of the strategy to construct plasmids used in this study. Only the relevant cloned gene(s) and restriction site(s) are shown. See Materials & Methods for further details.

were introduced into JMU193 according to standard procedures (Sambrook et al. 1989).

Recombinant DNA techniques. All general DNA manipulations were performed as previously described (Sambrook et al. 1989). Transformations of *E. coli* competent cells were carried out according to standard procedures (Sambrook et al. 1989).

Construction of pBTC1 and pBTC2 (Figure 2). pET101 was obtained by inserting the *Eco*RI-*Pst*I *phaC1* fragment from pGEc407 in pUC18. pET101 was then cut with *Eco*RI and *Hin*dIII to obtain a 2.2 kb fragment, which was cloned into the *Eco*RI and *Hin*dIII site of pJRD215, resulting in pET1 (Figure 2), or inserted into pVLT33, resulting in pBTC1. pET104 (Ren et al. 2000a) and pET103 contain a

B. Construction of *phaC2* plasmids

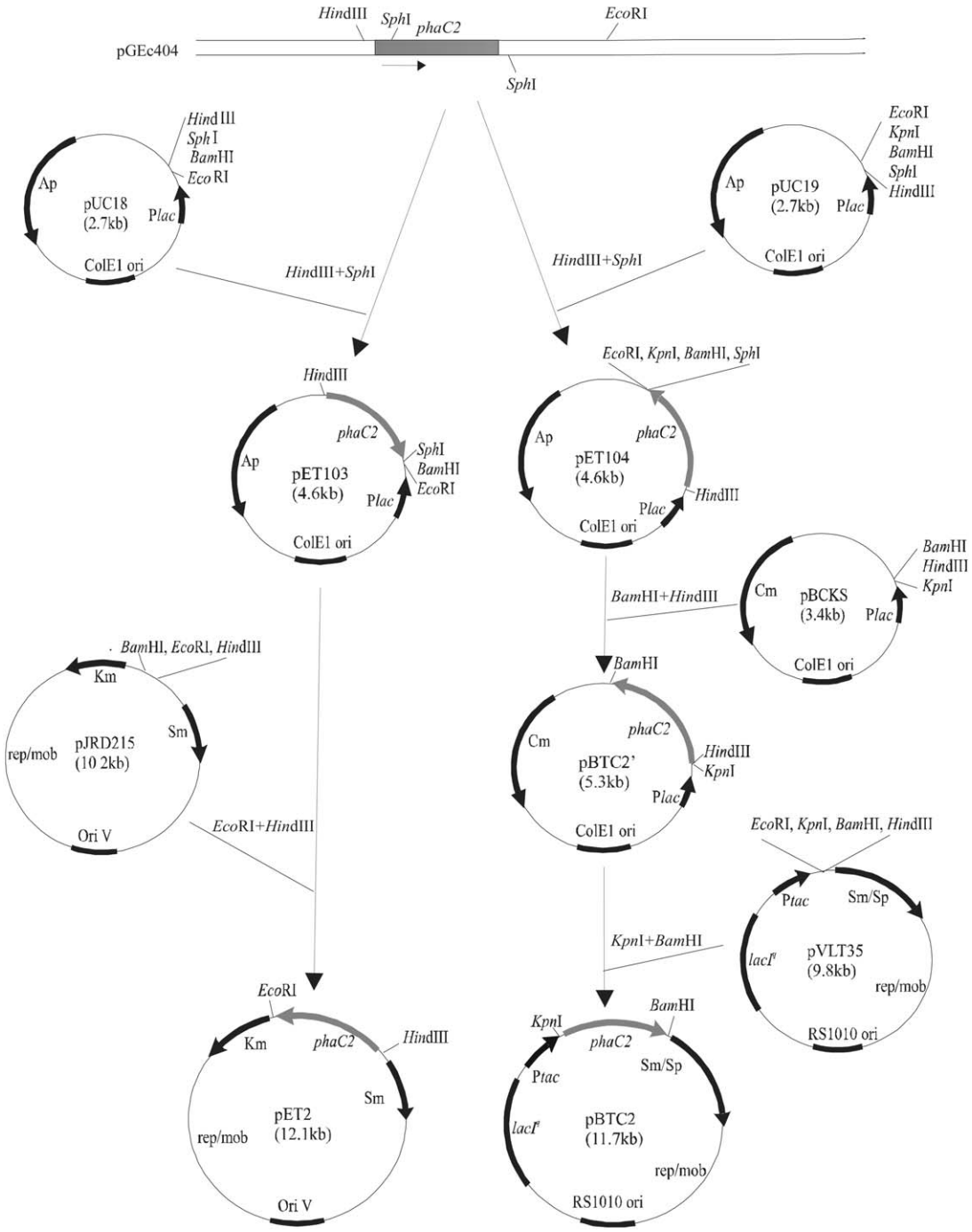


Figure 2. Continued.

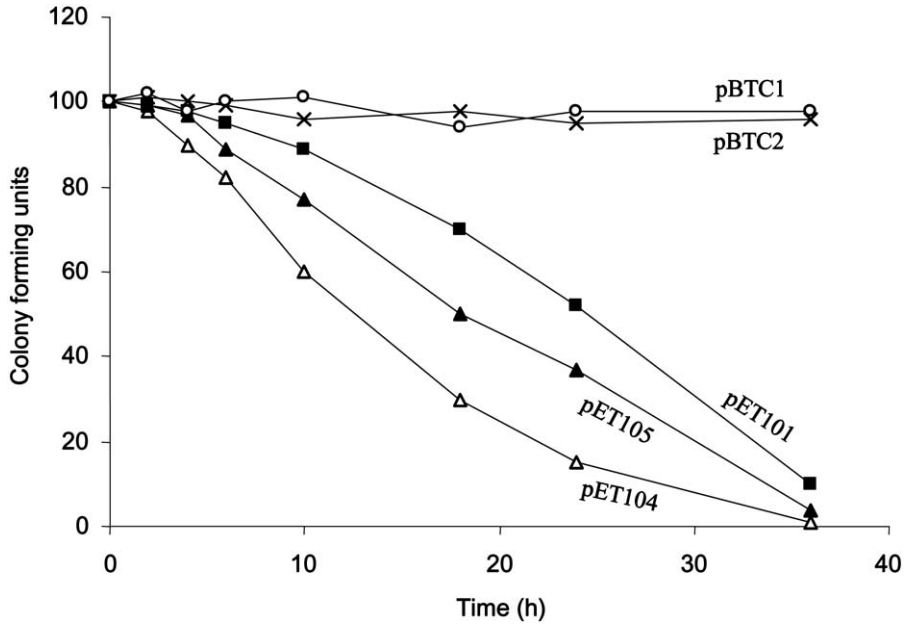


Figure 3. Stability of *phaC* containing plasmids in *E. coli*. *E. coli* recombinants JMU193 carrying pET101, pET104, pET105, pBTC1 and pBTC2 were grown on minimal media containing 2 mM hexadecanoate and 0.2% yeast extract with relevant antibiotics (ampicillin or kanamycin). Colony forming units were then monitored for 36 h.

*Hin*dIII-*Sph*I fragment with *phaC2* from pGEc404 in pUC19 and pUC18, respectively. pET105 was derived from pET104 by inserting an *Eco*RI excised *lacI*^{q} gene from pCNB5 into pET104. pET2 was obtained by inserting the *Eco*RI-*Hin*dIII fragment with *phaC2* from pET103 into pJRD215. pBTC2' (Ren et al. 2000a) was obtained by inserting *Bam*HI-*Hin*dII fragment of pET104 into pBCKS. pBTC2 (Ren et al. 2000a) contains the *Hin*dIII-*Bam*HI fragment with *phaC2* from pBTC2' in pVLT35.

Construction of pET2Ω. pMMB24 was cut with *Hin*dIII to obtain the transcriptional terminator Ω, which was then inserted into *Hin*dIII digested pET2, resulting in pET2Ω.

Media and culture conditions. *E. coli* was precultured at 37 °C in Luria-Bertani (LB) medium (Sambrook et al. 1989), then transferred with 1:100 dilution to 0.1NE2 (Huisman et al. 1989) minimal medium containing 2 mM hexadecanoate and 0.2% yeast extract as co-carbon source. Hexadecanoate stock solutions were prepared as previously described (Jenkins and Nunn 1987). If necessary, antibiotics were added: kanamycin (Km), 50 μg/ml, ampicillin (Ap), 150 μg/ml, streptomycin (Sp), 50 μg/ml, chloramphenicol (Cm), 30 μg/ml. Cell growth was monitored by measuring optical density at 450 nm (OD450) (Witholt 1972). In order to induce the P*lac* or P*tac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to indicated concentrations in the early exponential phase (OD450 about 0.2). Samples were taken as described in the results section.

Plasmid stability. To determine the percentage of cells that lost plasmids carrying the ampicillin, streptomycin or kanamycin resistance, cells were serially diluted and plated on LB plates with and without 150 μg/ml Ap, 50 μg/ml Sp or 50 μg/ml Km. The number of colonies was counted after overnight growth, and compared.

Determination of PHA. To determine the polyester content of bacteria, cells were grown in minimal medium cultures as indicated in the results section and assayed for the presence and composition of PHA by gas chromatography (GC) (Lageveen et al. 1988). All experiments were carried out 2 or 3 times, and averages of these independent experiments are presented.

Results and discussion

Previously we have reported that deficiency of the fatty acid degradation enzymes FadA or FadB enabled mcl-PHA synthesis in *E. coli* (Ren et al.

2000a). In this study, the *E. coli fadR fadB* mutant JMU193 was used. The *fadR* gene encodes a protein that exerts negative control over genes necessary for fatty acid oxidation (Black and DiRusso 1994; DiRusso and Nunn 1985; Jenkins and Nunn 1987; Nunn 1986). A mutation in *fadR* derepresses transcription of these genes, as a result of which the *fad* genes are constitutively expressed, rendering *E. coli* capable of growth on mcl fatty acids (Black and DiRusso 1994; DiRusso and Nunn 1985). The *fadB* gene encodes four enzyme activities of the fatty acid oxidation path: enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase (Black and DiRusso 1994; DiRusso and Nunn 1985; Jenkins and Nunn 1987; Nunn 1986). Mutations in *fadB* block fatty acid oxidation and result in the accumulation of specific intermediates, which can be channeled to PHA formation (Ren et al. 2000a).

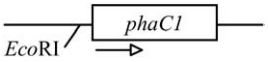
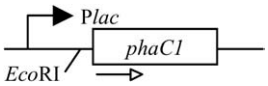
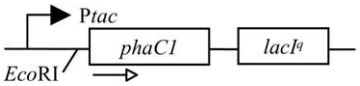
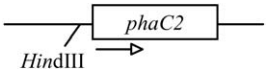
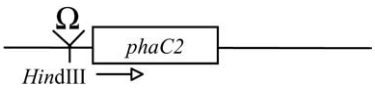
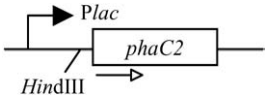
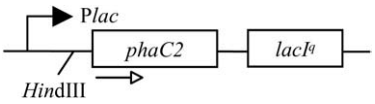
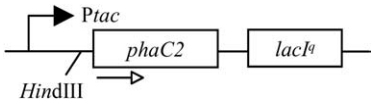
***PhaC1* and *phaC2* expression plasmids**. To better understand the consequences of regulated or unregulated *phaC1* and *phaC2* expression from their native or heterologous promoters in *E. coli*, we constructed the plasmids listed in Table 1. Plasmid pET1 contains the native, unmodified *phaC1* gene plus the upstream region (up to − 555 bp with respect to the ATG start codon) without external promoter (Figure 1). pET2 contains *phaC2* plus the upstream region (up to − 120 bp with respect to the ATG start codon) without external promoter (Figure 1). pET101 and pET104 contain *phaC1* and *phaC2*, respectively, with upstream regions and the genes are expressed from the P*lac* promoter (Table 1).

Expression of *phaC* genes and PHA formation in *pha*$^+$ *E. coli*. *E. coli* JMU193 was transformed with the plasmids described above. The transformants were cultivated in minimal medium containing 2 mM hexadecanoate and 0.2% yeast extract as described in 'Material and methods' and samples were analysed for PHA accumulation after 48 h. In *E. coli* JMU193[pET1], no trace of any of the methyl esters of 3-hydroxyalkanoates ($\leq 0.1\%$ PHA of cell dry weight) was found (Table 1), whereas in *E. coli* JMU193 harbouring pET101, a high copy number plasmid through which *phaC1* is constitutively expressed from the P*lac* promoter (i. e. the P*lac* promoter is not controlled by the monocopy *lacI* product), PHA accumulated to about 26% of the cellular dry matter (Table 1). Therefore, we conclude that the promoter of *phaC1* from *P. putida* GPo1 is not active in *E. coli*.

In *E. coli* JMU193 harbouring pET2, about 5% PHA of total cell dry weight was produced (Table 1), suggesting that a promoter upstream of *phaC2* is active in *E. coli*. To test this possibility, a transcriptional terminator was inserted into the polylinker region − 555 bp upstream of *phaC2* in pET2, resulting in pET2Ω (Table 1). No detectable PHA was found in *E. coli* JMU193 carrying pET2Ω. Thus, *phaC2* is not expressed in *E. coli* without an external promoter. The reason why 5% PHA was found in JMU193 carrying pET2 could be that a putative foreign promoter (such as promoters for antibiotics resistances) upstream of *phaC2* is present on pET2, in other words, the expression of *phaC2* on pET2 in *E. coli* is not driven by its native promoter but by a putative foreign promoter upstream of *phaC2*. Compared with pET2, pET1 did not lead to PHA production in recombinant *E. coli*, even though both *phaC1* from pET1 and *phaC2* from pET2 are located on the same plasmid. This could be caused by the reversed orientation of the two genes on the plasmid, resulting in that *phaC2* could be expressed by a putative foreign promoter, whereas *phaC1* could not. When *E. coli* JMU193 harbouring pET104 constitutive *phaC2* expression from the P*lac* promoter was tested, about 1.2% PHA was formed (Table 1).

Since each of the *phaC* genes from *P. putida* Gpo1 enables PHA synthesis in the PHA negative mutant *P. putida* GPp104, both genes are likely to have a similar function in the formation of PHA (Huisman et al. 1992, Huisman et al. 1991). It has been shown that there is an active promoter upstream of *phaC1* in *P. putida* (Huisman et al. 1991). A promoter upstream of *phaC2* has also been postulated for *P. aeruginosa* PAO1 (Timm and Steinbüchel 1992). Furthermore, expression of pET2Ω in *P. putida* GPp104 resulted in a PHA accumulation of 9.4% of the total cell dry weight, thereby proving the presence of an active promoter upstream of the *phaC2* gene. However, our results demonstrate that these two promoters are not active in *E. coli*. This is different from the well studied PHB synthetic genes, which are constitutively expressed from their own promoter in *E. coli* (Schubert et al. 1991). Since expression of many *Pseudomonas* genes is positively regulated, and these genes are not transcribed without the corresponding regulatory protein in *E. coli* (Deretic et al. 1989), our data indicates that not only the transcription of *phaC1* is regulated in *P. putida* GPo1, as reported previously (Prieto et al. 1999), but also probably that of *phaC2*. However, we cannot rule out other possibilities for the failure

Table 1. PHAs produced in *E. coli* JMU193 carrying different *phaC* expression plasmids

Plasmids[a]	Structure[b]	Vecors[b]	PHA%[c]	Monomer composition[d]		
				C6	C8	C10
pET1		pJRD215	≤ 0.1	–	–	–
pET101		pUC18	25.8	10	76	14
pBTC1*		pVLT33	17.3	11	78	11
pET2		pJRD215	4.8	6	88	6
pET2Ω		pJRD215	≤ 0.1	–	–	–
pET104		pUC19	1.2	–	–	–
pET105*		pUC19	10.8	7	76	17
pBTC2*		pVLT35	20.4	8	77	15

[a]*E. coli* recombinants were grown on 0.1NE2 with 2 mM hexadecanoate and 0.2% yeast extract. Samples were analyzed for PHA after 48 h. *, induced with 100 μM or high up to 1 mM IPTG at early exponential phase; [b]pJRD215 (low copy number), pVLT33/35 (low copy number) and pUC18/19 (high copy number) derived plasmids contain the *phaC1* or *phaC2* gene of *P. putida* GPo1 in orientations indicated by open arrows. The solid arrows represent the direction of the heterologous P*lac* or P*tac* promoter; [c]The amount of PHA is given as percentage of cell dry weight (cdw); [d]The monomer composition is given as molar percentage. C6, 3-hydroxyhexanoate; C8, 3-hydroxyoctanoate; C10, 3-hydroxydecanoate.

to obtain *phaC2* expression in *E. coli*, such as that the *phaC2* promoter is not recognised by the *E. coli* RNA polymerase.

Plasmid stability in *E. coli* recombinants expressing *phaC*. One possible reason for the lower amount of PHA associated with the high gene dosage in JMU193[pET104] compared to that in JMU193[pET2] could be the loss of the plasmid. Therefore, we investigated the stability of the constructed plasmids. To determine the percentage of cells that lost the antibiotic resistance, the JMU193 recombinants were grown in batch cultures in minimal media supplied with 2 mM hexadecanoate and 0.2% yeast extract in the presence of the selective marker (JMU193 carrying pET101 or pET104 with ampicillin, JMU193 carrying pET1 or pET2 with kanamycin), and the antibiotic resistance was monitored for approximately 36 h. Cultures were serially

diluted and plated on LB plates with and without 150 μg/ml ampicillin or 50 μg/ml kanamycin. The number of colonies was counted after overnight growth and compared. Plasmids pET1 and pET2 were 100% stable in JMU193 during the tested time period (data not shown). However, the presence of these plasmids in JMU193 resulted in no or low PHA production (Table 1). Figure 3 shows that in the JMU193[pET104] culture, 60% of the cells lost the ampicillin resistance within 10 h; while in the JMU193[pET101] culture, 89% of the cells remained ampicillin resistant after 10 h. The above results reveal that although both *phaC1* (in pET101) and *phaC2* (in pET104) were cloned in the same high copy number plasmid and equipped with the same strong promoter (Table 1), the latter is less stable in *E. coli* JMU193 (Figure 3). This difference is probably caused by the different upstream regions of *phaC1* in pET101 and *phaC2* in pET104 (Figure 1), or by the nature of the expressed protein.

To test whether uncontrolled expression of *phaC2* from pET104 plays a role in the plasmid instability, we constructed pET105 from pET104 (Table 1). pET105 overproduces the LacI repressor protein to control the expression of *phaC2*. Under induced conditions, about 77% of the JMU193[pET105] cells remained ampicillin resistant after 10 h growth in minimal media batch culture with hexadecanoate and yeast extract in the presence of ampicillin (Figure 3). To quantify the PHA content, JMU193[pET105] was cultivated on 0.1NE2 minimal medium with hexadecanoate and yeast extract as described in 'Materials and Methods'. About 11% PHA relative to cell dry weight was obtained from JMU193[pET105] after 48 h (Table 1), whereas less than 0.1% PHA was found without induction. Therefore, we conclude that uncontrolled overproduction of PHA polymerase in JMU103[pET104] is at least in part responsible for the observed low amount PHA. Regulated expression of *phaC2* from pET105 enhanced plasmid stability, which led to enhanced PHA accumulation. The loss of the PHA accumulation phenotype is possibly caused by the segregational instability of pET104 in *E. coli* JMU193, which could be further increased by the uncontrolled expression of *phaC2* from the strong heterologous P*lac* promoter on a high copy number vector: overproduction of PhaC2 might be toxic to *E. coli* cells. Subsequently, plasmid-free cells grew faster, allowing them to overgrow the recombinant cell population. This hypothesis was confirmed by using pET105. Although pET105 is also not completely stable in *E. coli* JMU193 (77% of the cells still contained plasmid after 10 hours), the increased stability compared to pET104 already allowed *E. coli* JMU193 to synthesize about 11% PHA upon IPTG induction.

Another reason for the instability of pET101, pET104 and pET105 in JMU193 could be the instability of ampicillin which is degraded in time by *E. coli* cells (Sambrook et al. 1989).

Application of stable plasmids in recombinant *E. coli*. The results described above show that the high copy number plasmids pET101, pET105 and pET104, all with ampicillin markers, were not stable enough for PHA production. This suggests that the combination of controlled *phaC* expression from a strong heterologous promoter on low copy number plasmid with another antibiotic marker might be effective in stabilizing PHA production in *E. coli*. Therefore, we constructed pBTC1 and pBTC2 (Figure 2), which are derived from a low copy number vector containing the P*tac* promoter, a *lacI*^{q} gene, and a streptomycin marker. As shown in Figure 3, both pBTC1 and pBTC2 are much more stable than pET101 and pET104 (or pET105) in *E. coli* JMU193 when the recombinants were grown in minimal media with hexadecanoate and yeast extract during the tested period. The plasmids were found to be structurally stable as well since the restriction patterns of plasmid DNA isolated during the cultivation were identical to those of the original constructs and they allowed re-transformed JMU193 to accumulate PHA (data not shown).

PHA accumulation using alternative *phaC* expression systems. *E. coli* JMU193 containing pBTC2 yielded more PHA than JMU193 containing pET104 or pET105, as shown in Table 1. The PHA content was increased from less than 1% without induction to about 20% in response to induction by 100 μM IPTG. A similar behaviour was found for JMU193 carrying pBTC1: no detectable PHA was found without induction, while about 17% PHA was formed when the recombinant was induced with 100 μM IPTG (Table 1). Higher IPTG concentrations (up to 1 mM) had no effect on PHA content or PHA monomer composition in both recombinants.

The PHA content in a host strain depends on the precursor concentration and the polymerase concentration (when it is below a certain level). The precursor concentration is presumably identical in *E. coli* recombinants JMU193 carrying pET2, pBTC2 and pET105. Since the PHA content (Table 1) differed in

these *E. coli* recombinants, these differences must be due to the relative polymerase concentrations. The higher amount of PHA accumulation in JMU193[pET105] (about 11% of cell dry weight) and JMU193[pBTC2] (about 20% of cell dry weight) than that found in JMU193[pET2] (about 5% of cell dry weight) suggests that higher *phaC* gene dosage can support the accumulation of PHA to a high concentration in recombinant *E. coli*, which has also been observed for poly-3-hydroxybutyrate (PHB) synthesis in *E. coli* (Lee and Chang 1995, Lee et al. 1994). In other words, relatively large amounts of enzyme seem to be required to direct the carbon flow from 3-hydroxyalkanoates to PHA synthesis among the several competing metabolic pathways such as continued ß-oxidation (Lee and Chang 1995). The reason why JMU193[pBTC1] produced less PHA (about 17% of cell dry weight) than JMU193[pET101] (about 26% of cell dry weight) might be the higher copy number of pET101 than pBTC1, although in both recombinants, *phaC1* is expressed from the strong promoters P*lac* or P*tac*. However, pET101 is not stable in recombinant *E. coli* (Figure 3), and thus not suitable for PHA production.

Effect of different *phaC* genes on the PHA composition. Table 1 shows that when both *phaC1* and *phaC2* genes are located on the same type of plasmid, the PHA polymers formed by PhaC1 consisted of more C6, similar amounts of C8 and less C10 (the molar ratio of C6:C8:C10 is 10:76:14 from pET101, 11:78:11 from pBTC1), compared to polymers produced by PhaC2 (the molar ratio of C6:C8:C10 is 7:76:17 from pET105, 8:77:15 from pBTC2). The PHA monomer composition depends on the precursor concentration, the specificity of the PHA polymerase for each precursor and the polymerase concentration (when it is below a certain level). The precursor concentration and the enzyme specificity profile are presumably identical in *E. coli* recombinants JMU193 carrying *phaC* genes. Since the two genes are located on the same type of vector, these PHA composition differences are not likely to be due to a gene dosage or a substrate concentration effect. Instead, these differences suggest that the polymerases have different substrate specificities. Evidently, PhaC1 has higher affinity to 3-hydroxyhexanoyl-CoA and lower affinity to 3-hydroxydecanoyl-CoA than PhaC2, as was also found for the PHA negative mutant *P. putida* GPp104 carrying each of these two *phaC* genes (Huisman et al. 1992).

Conclusion

In this study, we investigated the expression of two *phaC* genes from *P. putida* in *E. coli*. This led to the observation that significant PHA production in *E. coli* requires high *phaC1/C2* gene expression. However, the loss of the PHA accumulation phenotype occurred in *E. coli* recombinants when expression of *phaC* genes was not tightly controlled, which was similar to poly-3-hydroxybutyrate (PHB) production in *E. coli* (Lee et al. 1994). Apparently, *phaC*- or *phbCAB*-containing plasmids are lost when they are not vital to growth and impose detrimental effects on the cells. Therefore, for long term PHA production in *E. coli*, it is necessary to establish a system which allows stable and high PHA production. In the present report we generated a system where *phaC* expression was controlled by a strong heterologous promoter from low copy number plasmid and reached stable PHA production in *E. coli*. However, the PHA levels achieved in *E. coli* recombinants are not yet maximal, compared to that in native and recombinant *P. putida* where PHA is accumulated to 50-70% of cell dry mass (Huisman et al. 1992). One possibility is that the supply of PHA precursors is rate-limiting in *E. coli*, thereby limiting the attainable PHA production. Further experiments should be done to address this possibility.

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