Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant Escherichia coli grown on glucose

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Abstract

A recombinant Escherichia coli strain has been developed that produces poly(3-hydroxybutyrate-co-4-hydroxybutyrate) when grown in complex medium containing glucose. This has been accomplished by introducing into E. coli DH5α separate plasmids harboring the polyhydroxyalkanoate (PHA) biosynthesis genes from Ralstonia eutropha (formerly named Alcaligenes eutrophus) and the succinate degradation genes from Clostridium kluyveri, respectively. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) levels reached 50% of the cell dry weight and contained up to 2.8 mol.% 4-hydroxybutyrate. The molecular weight of the polymer was 1.8 × 10^6. © 1997 Elsevier Science B.V.

Keywords: Polyhydroxyalkanoate; Succinate degradation; Ralstonia eutropha; Clostridium kluyveri

1. Introduction

Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] is a recently discovered PHA that has attracted considerable interest because of its increased flexibility in thermoplastic applications (Kunioka et al., 1989; Doi et al., 1990a,b, Saito and Doi, 1994). Generally, P(3HB-co-4HB) is produced by feeding precursors such as 3-hydroxybutyrate, 1,4-butanediol or γ-butyrolactone to wild type or mutant strains of Ralstonia eutropha. More recently, Doi has described a method whereby γ-butyrolactone can be fed to Alcaligenes latus to obtain P(3HB-co-4HB) levels that are ≈ 60% of the cell dry weight and which contain 7–12 mol.% 4-hydroxybutyrate (4HB) monomers (Soejima and Doi, 1996). Normally the molar levels of 4HB in the copolymer are relatively low, but recently R. eutropha mutants have been isolated which were able to accumulate a 4HB homopolymer. When these mutants were supplemented with the R. eutropha PHA synthase gene the 4HB homopolyester was accumulated to
levels of \( \approx 30\% \) of the cell dry weight (Steinbüchel et al., 1994).

Studies have indicated that the pathway for 4HB metabolism in \( R. \) eutropha occurs by conversion to succinic semialdehyde via a 4-hydroxybutyrate dehydrogenase and subsequent conversion of succinic semialdehyde to succinate by a succinic semialdehyde dehydrogenase (Valentin et al., 1995). The metabolic link to P(3HB-co-4HB) formation was not elucidated in detail, but the presence of high activities of 3HB-dehydrogenase, an enzyme of the intracellular PHA degradation pathway, in crude extracts of 4HB-induced \( R. \) eutropha led the authors to suggest that 4-hydroxybutyryl-CoA (the immediate precursor before polymerization) might be supplied by the action of a succinyl-CoA:acetoacetate CoA transferase, or an undefined thiokinase; and that 3-hydroxybutyryl-CoA is supplied by the concerted action of the TCA-cycle, gluconeogenesis and the PHA biosynthesis enzymes.

Recently a similar pathway, involved in the cofermentation of succinic acid and ethanol in \( C. \) kluyveri has been described (Söhling and Gottschalk, 1996) in which 4-hydroxybutyryl-CoA is an intermediate. In this pathway, CoA is transferred to succinate via the action of an enzyme having succinyl-CoA transferase activities. Succinyl-CoA is then reduced to succinic semialdehyde and CoA via a succinic semialdehyde dehydrogenase. Succinic semialdehyde is reduced to 4HB (via a 4-hydroxybutyrate dehydrogenase) which is then activated to 4-hydroxybutyryl-CoA by an enzyme possessing 4-hydroxybutyryl-CoA:CoA transferase activity. The genes for this pathway have been located on a 7.5 kb DNA fragment that includes genes for 4-hydroxybutyrate dehydrogenase (4hbD), succinic semialdehyde dehydrogenase (sucD), succinyl-CoA:CoA transferase (cat1), a membrane protein of unknown function (orfY) and 4-hydroxybutyryl-CoA:CoA transferase (designated as orfZ in original paper). GenBank™ accession number L21902. Furthermore, three of the proteins, 4HBD, SUCD and CAT1 were found to be expressed in \( E. \) coli.

It is already well established that the PHA biosynthesis operon from \( R. \) eutropha is sufficiently expressed in \( E. \) coli to mediate PHB production to levels as high as 70–80% of the cell dry weight (Schubert et al., 1988; Slater et al., 1988; Peoples and Sinskey, 1989). Therefore, introduction of the sucD, 4hbD and orfZ genes from \( C. \) kluveri into a recombinant \( E. \) coli strain containing the PHA biosynthesis genes could facilitate the production of 4-hydroxybutyryl-CoA for incorporation into P(3HB-co-4HB) (Fig. 1). Moreover, this incorporation would not depend on immediate precursors of 4-hydroxybutyrate, but should occur simply from abstraction of succinate or succinyl-CoA from the citric acid cycle when the bacterial strain is grown on glucose. This paper describes experiments which suggest that this is the case.

![Proposed pathway to poly(3HB-co-4HB) synthesis in recombinant \( E. \) coli harboring plasmids carrying the PHA synthesis operon from \( R. \) eutropha and the succinate utilization pathway from \( C. \) kluveri. Enzymes: 1, 3-ketothiolase; 2, acetoacetyl-CoA reductase; 3, PHA synthase, 4, succinic semialdehyde dehydrogenase; 5, 4-hydroxybutyrate dehydrogenase; 6, 4-hydroxybutyryl-CoA:CoA transferase.](image-url)
2. Materials and methods

2.1. Bacterial strains and plasmids

_E. coli_ DH5α-F-φ80dlacZAM15Δ(lacZYA-argF)U169 deoR recA1 endA1 phoA hsdR17 (rK-, mK+)
were used as the host (Gibco-BRL, Bethesda, MD). The plasmid pJM9238 is a runaway replication
vector containing the genes for the poly(3-hydroxybutyrate) biosynthesis pathway from _R. eutropha_
and has been previously described (Kidwell et al., 1995).

2.2. Plasmid construction

pCKS carries the genes for the succinate degradation pathway from _C. kluyveri_ and is a derivative of pCK3 which has previously been described (Söhling and Gottschalk, 1996). To make pCKS, the plasmid pCK3 was subjected to partial _EcoRI_ digestion and a 9.4 kb fragment was excised from a gel and ligated. This plasmid, pCK3A contained the genes for the succinate degradation pathway minus a 1.1 kb _EcoRI_ fragment which encodes a putative membrane spanning protein of unknown function and the amino terminal region of _cat1_ (succinyl-CoA:CoA transferase). The insert that remained was removed by digesting with _ApaI_ and _SacI_ and was cloned back into pBlueScript II-SK (Stratagene, La Jolla, CA) such that the _lacZ_ promoter promoted transcription in the same direction as the three intact genes on the insert (4hbD, sucD, orfZ).

2.3. Cell growth

Bacterial strains used in these experiments were cultured overnight in 16 × 100 mm sterile tubes containing 3 ml of LB and the appropriate antibiotics (chloramphenicol at 25 µg ml⁻¹ for pJM9238, ampicillin at 100 µg ml⁻¹ for pCKS). A 1 ml volume of the overnight culture was used to inoculate 50 ml of the same media in 250-ml baffled shake flasks. The cultures were grown at 30°C until they reached an optical density of 0.7 (600 nm), at which time glucose was added to a final concentration of 1% (w/v) and the cultures were shifted to an orbital incubator that was at 37°C to induce pJM9238 into runaway replication. Cells were harvested after ≈ 48 h of incubation at 37°C.

2.4. PHA analysis

Cells were harvested by centrifugation at 3500 rpm (Heraeus Varafuge F) followed by suspension of the pellet in 0.9% (w/v) saline and recentrifugation to obtain a washed pellet. The washed pellet was frozen and dried by lyophilization. Intracellular polyhydroxyalkanoate was characterized using a previously described modification (Slater et al., 1992) of the gas chromatographic method developed by Braunegg et al. (1978). P(3HB) (Sigma Chemicals) and γ-butyrolactone were used as standards.

2.5. Extraction of the polymer

The polymer was extracted by shaking the dried bacterial cell pellet overnight at 37°C in chloroform, removing the bacterial debris by filtration of the chloroform/bacterial suspension through Whatman paper and precipitating the polyester from the filtered solution by the addition of 10 volumes of ethanol. The precipitated polymer was redissolved in chloroform, reprecipitated with 10 volumes of methanol, filtered onto a Whatman paper filter and allowed to dry. The polymer was redissolved in deuterochloroform or chloroform and used for nuclear magnetic resonance and gel permeation chromatography analysis, respectively.

2.6. 1H and 13C{1H} NMR analyses

Nuclear magnetic resonance studies were carried out using a Bruker AC/200 spectrometer. The 1H spectrum (200 MHz) was obtained at 22°C on a solution of polymer that was dissolved at a concentration of ≈ 20 mg ml⁻¹ in deuterochloroform. Pulses were taken using a 45° pulse width, 2.46 s acquisition time, 3333 Hz spectral width, 16 k data points and 64 accumulations. Chemical shifts were measured relative to CHCl₃ (δ = 7.24 ppm). The 13C{1H} spectra (50 MHz) were taken
Table 1
Poly(3HB-co-4HB) accumulation in recombinant E. coli

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>IPTG (mM)</th>
<th>Mg cells</th>
<th>Mol.% 3HB</th>
<th>Mol.% 4HB</th>
<th>%P(3HB-co-4HB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJM9238</td>
<td>0</td>
<td>84</td>
<td>100.0</td>
<td>0.0</td>
<td>52</td>
</tr>
<tr>
<td>pJM9238, pCKS</td>
<td>0</td>
<td>26</td>
<td>98.5</td>
<td>1.5</td>
<td>46</td>
</tr>
<tr>
<td>pJM9238, pCKS</td>
<td>5</td>
<td>20</td>
<td>98.7</td>
<td>1.3</td>
<td>45</td>
</tr>
</tbody>
</table>

at 22°C on a polymer solution dissolved in deuterochloroform at \( \approx 50 \text{ mg ml}^{-1} \). The spectra were obtained using Waltz decoupling, 30° pulses, 1 s relaxation delay, 12 kHz spectral width, 32 k data points and 93 000 accumulations. Chemical shifts were measured relative to CDCl₃ (\( d = 77.0 \text{ ppm} \)).

2.7. Gel permeation chromatography

Molecular weight analyses were carried out employing gel permeation chromatography as previously described by Koizumi et al. (1995).

3. Results and discussion

3.1. Gas chromatographic analysis of PHA produced in E. coli (pJM9238, pCKS)

*E. coli* (pJM9238) and *E. coli* (pJM9238, pCKS) were cultured in LB containing glucose to promote PHA synthesis and provide substrates for glycolysis and the TCA cycle. The cells were collected 48 h after induction of PHA accumulation, dried and analyzed for the PHA polyesters (Table 1). Even though an extremely large amount of dried cell pellet from the *E. coli* (pJM9238) culture was analyzed (84 mg), no 4HB monomers could be detected as constituents of the polymer. The overall level of PHA (52% of cell dry weight) was consistent with previous experiments using this vector and host (Kidwell et al., 1995). When *E. coli* (pJM9238, pCKS) was analyzed (carrying the PHA biosynthesis genes and the succinate degradation pathway genes), very small peaks could be seen on the chromatogram (data not shown) that corresponded exactly to the retention times of the 4HB standard. These were taken to be 4HB. The final level of 4HB in the polymer was 1.5 mol.%. To determine if the succinate degradation genetic pathway on pCKS might be under control of the *lac* promoter upstream of the genes, the experiment was also carried out in the presence of IPTG (added at the time of temperature upshift) to induce the *lac* promoter. The level of 4HB in the polyester was virtually unchanged (1.3 mol.%), indicating that the *lac* promoter probably does not play a role in the expression of the succinate degradation genes. When the original plasmid, pCK3 (which contains the succinate degradation pathway of *C. kluyveri* in the opposite orientation from the *lac* promoter) was used in a similar experiment, only 0.2 mol.% 4HB was found in the polymer. When the DNA insert in pCK3 (containing the entire succinate degradation pathway) was reversed, thereby placing the *lac* promoter colinear with the succinate degradation genes, the level of 4HB was 0.3 mol.% (data not shown). These results suggest that the presence of an intact succinyl-CoA:CoA transferase gene does not mediate a measurable increase in 4HB levels, as might be expected, but appears to result in a decrease in 4HB levels (in comparison to *E. coli* (pJM9238, pCKS)). However, this cannot be considered strong evidence since succinyl-CoA:CoA transferase activity was not measured and the contribution of the orfY gene product is unknown.

3.2. NMR analysis of the purified polymer

Because the level of 4HB in the polymer was quite low, confirmation of its presence was sought by nuclear magnetic resonance spectrometry of the purified polymer. For NMR analysis a larger culture (500 ml) of *E. coli* (pJM9238, pCKS) was grown under conditions described in Section 2 except that the culture contained 2% glucose. The
culture yielded 439 mg of cell dry mass from which 115 mg of P(3HB-co-4HB) was extracted. This polymer was subjected to both $^1$H and $^{13}$C{$^1$H} NMR spectroscopy (Fig. 2). Both spectrographs displayed a pattern of signals virtually identical to spectra of P(3HB-co-4HB) that have been previously published (Doi et al., 1988; Steinbüchel et al., 1994). Signals 6, 7 and 8 represent the resonances of the corresponding 4HB-methylene groups (Fig. 2A). The $^{13}$C{$^1$H} NMR signals of the 4HB carbonyl carbon (signal 5) and the methylene carbons (signals 6, 7 and 8) can be seen in Fig. 2B at 171.9, 30.8, 24.0 and 63.7 ppm, respectively, Using the integration data from the $^1$H NMR, we were able to accurately determine the composition of the polymer, which was 97.2 mol.% 3HB and 2.8 mol.% 4HB. As in the gas chromatographic analysis no other signals were seen that could be interpreted to be additional polyhydroxyalkanoates resident in the polymer. It is important to note that the 4HB produced is done so, in spite of the deletion of the succinyl-CoA:CoA transferase gene which is necessary for succinate degradation in C. kluyveri. This is almost certainly due to the high flux of intracellular succinyl-CoA and its availability, through the TCA cycle.
3.3. Molecular weight of P(3HB-co-4HB) from E. coli (pJM9238, pCKS)

To determine if the addition of 4HB units into the polymer in E. coli might have detrimental effects on the molecular weight, a portion of the extracted polymer isolated for the NMR experiment was subjected to gel permeation chromatography. The estimated molecular weight of this polymer was 1.8 × 10^6. Previous analyses of poly(3-hydroxybutyrate) extracted from recombinant E. coli (pJM9238) had shown that the molecular weight of the polymer usually was in the range of 1.0 to 3.0 × 10^6 (Kidwell et al., 1995; unpublished data) so it does not appear that the incorporation of 4HB into the polymer at these levels causes any significant decrease in the molecular weight. Based on these and the other experiments above, we surmise that it is possible to produce a high molecular weight polymer of P(3HB-co-4HB) in recombinant systems.

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References


