Polyhydroxyalkanoate production in recombinant *Escherichia coli*

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1. SUMMARY

The bacterial species *Escherichia coli* has proven to be a powerful tool in the molecular analysis of polyhydroxyalkanoate (PHA) biosynthesis. In addition, *E. coli* holds promise as a source for economical PHA production. Using this microorganism, clones have been developed in our laboratory which direct the synthesis of poly-β-hydroxybutyrate (PHB) to levels as high as 95% of the cell dry weight. These clones have been further enhanced by the addition of a genetically mediated lysis system that allows the PHB granules to be released gently and efficiently. This paper describes these developments, as well as the use of an *E. coli* strain to produce the copolymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-3HV).

2. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are commonly found in many different species of bacteria, particularly those that are considered to be soil microorganisms [1]. It appears that the polymer acts as a mechanism for storage of energy during times in which carbon levels are high, but other essential nutrients, such as nitrogen, are limiting. Researchers have taken advantage of this fact in the laboratory, and commercially, to produce PHAs to very high intracellular levels [2]. The types of PHA polymers, and the levels at which they are made, vary greatly depending on the bacterial species, culture conditions, and carbon source [1,2]. Of particular interest is recent work that has shown that some bacterial species are capable of synthesizing higher-order polyhydroxyalkanoates using relatively simple carbon sources as substrates [3–5]. Nevertheless, before PHAs can make the successful move from the laboratory to the commercial marketplace, technologies must be developed that decrease the cost of production.

A primary aid in this goal has been the cloning and study of the poly-β-hydroxybutyrate biosynthetic pathway from *Alcaligenes eutrophus* [6–8]. This cloning implemented the elucidation of the transcriptional strategy of the PHB biosynthetic pathway [9], as well as the determination of the three-gene DNA sequence [8–11]. The cloning of the PHB biosynthetic genes has also resulted in the construction of *E. coli* strains that accumulate PHB to levels as high as 95% of the cell dry weight [11], as well as the construction of trans-
genic plants that accumulate small amounts of PHB [12].

Because of the need for a more economical source of PHB, our laboratory has sought to apply the power of E. coli genetics to this particular PHB production system, especially in the areas of rapid accumulation of PHB to high intracellular levels and economical methods of purification of PHB granules. In addition, we have used E. coli mutants to produce the copolymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-3HV). This paper reports several of these advances.

3. RESULTS AND DISCUSSION

In our initial plasmid constructs that contained the PHB biosynthetic genes, we were not able to achieve intracellular PHB levels much above 50% (polymer weight/dry cell weight). This was also found to be the case in two separate laboratories [7,8]. However, in the course of producing ‘nested-set’ deletions for DNA sequence analysis one plasmid was isolated that exhibited superior PHB producing abilities. This plasmid was designated p4A. When compared to the PHB biosynthetic pathway cloned into other high copy number vectors, p4A directed the synthesis of PHB to significantly higher levels (Fig. 1). In further experiments in both shake-flasks and fermentors, E. coli strains harboring p4A have consistently accumulated PHB to greater than 90% of the cell dry weight (Fig. 2).

Experiments have shown that this high level of PHB production is almost certainly derived from a gene dosage effect. Alkaline lysate minipreps of p4A normally produce two to three times as much plasmid DNA as normal minipreps, and copy number experiments have demonstrated that p4A has a copy number of approximately 500 in the stationary phase of the bacterial culture. Moreover, in experiments where the PHB pathway was cloned into runaway expression vectors, intracellular PHB levels were very low (less than 10%) when the basal copy number was held to 1, whereas when the vector was induced into runaway replication the PHB levels increased to lev-

3.1. Release of PHB granules

In previous work, it has been shown that it is possible to release the PHB granule from the E.

Fig. 1. Comparison of intracellular PHB accumulation in E. coli DH5alpha strains containing (•) pUC13/PHB, (▲) pGEM7f/PHB and (■) p4A.

Fig. 2. Scanning electron micrograph of E. coli cells in which the cell wall has been collapsed around intracellular PHB granules. PHB accumulation in this preparation was approximately 95% PHB (polymer weight/cell dry weight). Magnification is approximately 4000×.
coli cell using genetically mediated lysis [13]. In this system, a bacteriophage lysis gene under the control of a temperature-regulated promoter was derepressed by heating the culture, allowing the production of the lysis protein and the subsequent lysis of the cell. Employing this strategy, PHB granules are released from the cell in a gentle and economical manner. However, the system suffered from the fact that the bacteriophage protein was not active in stationary-phase bacterial cells, the phase in which most PHB accumulation takes place. Hence, efficient lysis could only be obtained during exponential growth prior to the time when maximal PHB accumulation was obtained.

Therefore, we have developed another system which uses a separate plasmid that contains the T7 bacteriophage lysozyme gene expressed at low levels throughout the cell cycle [14]. During the growth cycle, the lysozyme does not penetrate the inner membrane and does not disrupt the integrity of the peptidoglycan layer. However, at the end of the PHB accumulation phase of the culture, the cells are pelleted and resuspended in the chelating agent ethylenediaminetetraacetic acid (2 mM EDTA in 50 mM Tris buffer, pH 8), disrupting the integrity of the inner membrane. At this point the lysozyme gains access to the structural peptidoglycan layer and digests it. Triton X-100 at a final concentration of 0.1% (wt/vol) is added to the cells and the weakened cells burst, usually at their polar caps, releasing the PHB granules (Fig. 3). The efficiency of lysis is greater than 99%. Interestingly, scanning electron micrographs of this phenomenon indicate that the PHB granules are considerably larger than the empty E. coli ‘ghosts’ that are left behind. This seems to imply that the E. coli cells have a genetically predetermined size, but can be stretched to accommodate intracellular inclusions larger than this predetermined size.

Because of the potentially detrimental effect the intracellular lysozyme might have on cell growth and PHB accumulation, we conducted several experiments in shake-flasks comparing the p4A non-lysis strain with the lysis-proficient strain (Fig. 4). In all experiments, the lysis-proficient strain produced substantially greater amounts of PHB than the non-lysis. In fact, in one experiment the PHB levels approached 15 g of PHB per liter of culture (data not shown). To our knowledge, this is the highest PHB production ever reported in shake-flasks, and compares favorably with PHA yields reported in many fermentations.
Released PHB granules may be purified from the lysed cell debris in a number of ways, but the most efficient strategy appears to be selective precipitation by the addition of a hardening agent such as calcium chloride. In preliminary small-scale experiments we have been able to selectively aggregate PHB particles, causing them to fall to the bottom of the test tube. Further experiments are being conducted.

3.2. Production of PHB-co-3HV in E. coli

The increased flexibility of PHB-co-3HV films over PHB films is well known and commercialization efforts have focused on this particular polyhydroxyalkanoate [2]. Because of this commercial importance and because of our interest in the genes that control copolymer synthesis, we initiated experimentation aimed at obtaining copolymer production in an *E. coli* system. Based on previous research [15–18] we reasoned that the propionyl-CoA necessary for hydroxyvalerate production must come from an inducible enzyme system, possibly one that could be induced by acetate and/or propionate. In preliminary experiments we attempted to induce the putative enzyme(s) of propionyl-CoA synthesis by adapting PHB-positive *E. coli* strains to growth on acetate, followed by the addition of propionate and glucose to initiate PHB-co-3HV synthesis [19]. This strategy worked, but only marginally in that small amounts of 3-hydroxyvalerate (3HV) were incorporated into the copolymer. In further experiments we employed PHB-positive *E. coli* strains that were *fadR, atoC(Con)* mutants. These mutations cause the constitutive expression of fatty acid utilization enzymes. In experiments where the bacteria were grown in minimal medium containing 2% glucose and 25 mM propionate we consistently obtained hydroxyvalerate incorporation levels of 20–30 mol% of the total polymer. The amount of 3HV incorporation can be controlled by regulating the amount of propionate in the culture (Fig. 5), the amount of glucose in the culture, the time of propionate addition, and the type of medium used. At lower propionate levels (below 10 mM), the efficiency of incorporation into 3HV approaches 100%. At higher propionate levels, the efficiency of incorporation drops considerably and it appears that the highest level of 3HV incorporation attainable before the high propionate levels become toxic to the cells is 50 mol%. The method of copolymer production, and the genes which control this production, are currently being studied.

3.4. Conclusion

*E. coli* has proven to be a versatile performer not only in expediting the molecular analysis of PHA biosynthesis, but also in (1) synthesizing the biopolymer to extremely high intracellular levels, (2) being amenable to specific genetic strategies such as genetically mediated lysis, and (3) the utilization of mutants to metabolically engineer a strain that produces PHB-co-3HV copolymer. Given the vast amount of knowledge concerning *E. coli* genetics, and the increasing amount of knowledge concerning the metabolic events necessary for polyhydroxyalkanoate synthesis, we fully expect that *E. coli* will continue to play a significant part in the determination of the mechanisms of PHA synthesis and, possibly, the commercial production of PHAs.
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REFERENCES