JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4431–4436
0021-9193/88/104431-06$02.00/0

Vol. 170, No. 10

Cloning and Expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 Poly-β-Hydroxybutyrate Biosynthetic Pathway

STEVEN C. SLATER,[1] WILLIAM H. VOIGE,[2] AND DOUGLAS E. DENNIS[1]*

Departments of Biology[1] and Chemistry,[2] James Madison University, Harrisonburg, Virginia 22807

Received 31 July 1987/Accepted 5 July 1988

The poly-β-hydroxybutyrate (PHB) biosynthetic pathway from *Alcaligenes eutrophus* H16 has been cloned and expressed in *Escherichia coli*. Initially, an *A. eutrophus* H16 genomic library was constructed by using cosmid pVK102, and cosmid clones that encoded the PHB biosynthetic pathway were sought by assaying for the first enzyme of the pathway, β-ketothiolase. Six enzyme-positive clones were identified. Three of these clones manifested acetoacetyl coenzyme A reductase activity, the second enzyme of the biosynthetic pathway, and accumulated PHB. PHB was produced in the cosmid clones at approximately 50% of the level found in *A. eutrophus*. One cosmid clone was subjected to subcloning experiments, and the PHB biosynthetic pathway was isolated on a 5.2-kilobase *Kpn*I-*Eco*RI fragment. This fragment, when cloned into small multicopy vectors, can direct the synthesis of PHB in *E. coli* to levels approaching 80% of the bacterial cell dry weight.

Poly-β-hydroxybutyrate (PHB), a homopolymer of D-(−)-3-Hydroxybutyrate, is a storage material produced by a variety of bacteria in response to environmental stress. The presence of PHB in bacteria was first recognized by Lemoigne in 1926 (11), and it has since been identified in more than 20 bacterial genera, including *Azotobacter*, *Bacillus*, *Beijerinckia*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, and *Rhodospirillum* (4).

The PHB pathway and its regulation have been studied extensively in *Alcaligenes eutrophus* H16 and *Azotobacter beijerinckii* (3, 4, 9, 10, 15, 18–21, 24). The pathway consists of a biosynthetic portion and a degradative portion and is made up of five enzymes. One of these enzymes, β-ketothiolase, is both the entry point and the exit point of the cycle (4). In the biosynthetic part of the pathway, β-ketothiolase catalyzes the reversible condensation of two acetyl coenzyme A (CoA) molecules to acetoacetyl-CoA. Acetoacetyl-CoA is subsequently reduced to D-(−)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase, and PHB is then produced by the polymerization of β-hydroxybutyryl-CoA via the action of PHB synthetase.

Studies in *A. eutrophus* H16 and in *Azotobacter beijerinckii* have shown the PHB pathway to be regulated in response to several types of environmental limitation. These limitations include oxygen deprivation, nitrogen deprivation, sulfate limitation, and magnesium limitation (3, 4, 10, 15, 18–21, 24). Under limiting environmental conditions, PHB may constitute as much as 80% of the dry cell weight. When limiting conditions are relaxed, PHB quantities decrease to preinduction levels (4). Induction studies in which β-ketothiolase and acetoacetyl-CoA reductase were studied have revealed that both enzymatic activities increase markedly in response to PHB-stimulating limitations (4, 10, 15, 19).

These experiments indicate that the PHB pathway may exhibit a mode of transcriptional control that is similar to that of other metabolic pathways that are induced by environmental stress. Examples of such pathways include the heat shock regulon, the *pho* regulon, and the carbon starvation regulon. Therefore, analysis of the PHB biosynthetic pathway may aid in elucidating the mechanisms by which these global regulatory networks operate. In this paper, we discuss initial experiments conducted on the PHB pathway of *A. eutrophus* H16. These experiments include the cloning of the PHB biosynthetic pathway and the production of PHB in *Escherichia coli* to a high internal concentration.

* Corresponding author.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo., or from United States Biochemicals, Cleveland, Ohio. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or from United States Biochemicals. Agarose was Seakem GT agarose from FMC Corp., Marine Colloids Div., Rockland, Maine.

Bacterial strains and media. *A. eutrophus* H16, *E. coli* LE392, and *E. coli* DH1 were obtained from the American Type Culture Collection. *E. coli* DH5 and plasmid pUC18 were obtained from Bethesda Research Laboratories. Luria broth (LB) and antibiotics were prepared by the method of Maniatis et al. (13). The cosmid pVK102 was obtained in *E. coli* HB101 from the American Type Culture Collection. The plasmid pUC13 was obtained from Pharmacia.

Cosmid library construction. Total *A. eutrophus* H16 DNA was extracted by the sarcosyl lysis method (17). A series of partial *Sal*I digests of the DNA was conducted in order to determine the reaction conditions that would yield the maximum percentage of DNA fragments in the 20- to 25-kilobase (kb) range. By using these parameters, a large-scale digest was performed and the DNA was purified by phenol extraction and ethanol precipitation. The cosmid pVK102 was extracted by the method of Hansen and Olsen (8), purified in a CsCl gradient, digested with *Sal*I, and purified by phenol extraction and ethanol precipitation. The partially digested genomic DNA fragments and the cosmid were mixed at an insert-to-vector molar ratio of 20:1 at a final total DNA concentration of approximately 400 μg/ml, and the mixture was subjected to ligation overnight at 14°C. Part of the ligation was packaged by using the Promega Packagene kit, and the packaged cosmids were used to transfect *E. coli* LE392. The bacteria were plated onto plates of LB plus kanamycin, and resultant clones were picked for use in the library. Clones were stored individually in LB plus 15% glycerol at −85°C.

TABLE 1. Analysis of cosmid clones for enzyme activity and PHB accumulation

Bacterium[a]	Cosmid	β-Ketothiolase activity[b]	Acetoacetyl-CoA reductase activity[c]	mg of PHB/ml of culture	% PHB
E. coli LE392	None	0.0	0.0	0.0	0
A. eutrophus H16	None	12.4	12.3	1.18	35
E. coli LE392	pAE65	5.2	39.0	0.03	1
E. coli LE392	pAE175	16.2	0.2	0.47	16
E. coli LE392	pAE537	2.4	0.0	0.0	0
E. coli LE392	pAE683	10.4	0.0	0.0	0
E. coli LE392	pAE689	14.8	0.2	0.64	20
E. coli LE392	pAE902	8.5	0.0	0.0	0

[a] Bacteria were grown in LB plus 1% gluconate.
[b] Micromoles of acetoacetyl-CoA degraded per minute per milligram of protein.
[c] Micromoles of NADPH reduced per minute per milligram of protein.

Preparation of cell extracts for enzyme assay. One milliliter of an overnight culture in LB was pelleted by centrifugation in a microcentrifuge for 1 min. The supernatant was removed, and the pellet was resuspended in 200 μl of breaking buffer (20 mM potassium phosphate buffer [pH 7.2], 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 M glycerol). The suspension was subjected to sonication by using an Artek 300 sonicator with a microprobe at the maximum setting. Sonication consisted of four 15-s bursts. The sonic extract was subjected to centrifugation in a microcentrifuge for 5 min, and the supernatant was transferred to a different microcentrifuge tube on ice for analysis. For assays done at later times, the cells were pelleted by centrifugation in a microcentrifuge at room temperature for 1 min, the supernatants were removed, and the pellets were stored at −85°C until assay, at which time the pellets were resuspended and sonicated as described above.

Enzyme assays. β-Ketothiolase assays (thiolysis reaction), acetoacetyl-CoA reductase assays, succinyl-CoA transferase assays, and D-3-hydroxybutyrate dehydrogenase assays were conducted by using the methods of Senior and Dawes (19, 20).

Protein determination. Protein was measured by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

PHB assay. The PHB assay was done by the procedure of Ward and Dawes (23), except that Whatman GF/F filters were used instead of Whatman GF/A filters. PHB amounts were calculated from a standard curve by using known quantities of DL-hydroxybutyrate.

Southern blot hybridization. Southern blot analysis was performed by the method of Maniatis et al. (13). The probe was made radioactive by using a random primer extension kit obtained from DuPont, NEN Research Products, Boston, Mass.

Subcloning of cosmid and plasmid DNA fragments. Recombinant cosmids were purified by the method of Hansen and Olsen (8). The purified recombinant cosmid was digested with the appropriate restriction endonuclease, and the fragments to be cloned were isolated in low-melting-temperature agarose (2). Ligation reactions contained plasmids and insert DNA at a 1:3 ratio, respectively.

IR spectra. The infrared (IR) spectra of various PHB samples were obtained by using the technique of Wakisaka et al. (22).

RESULTS

Generation and initial screening of the *A. eutrophus* H16 library. A cosmid library of *A. eutrophus* H16 total DNA was constructed by inserting 20- to 25-kb DNA fragments into pVK102, followed by transduction of *E. coli* LE392. Approximately 1,100 clones were picked for further assay. Of these clones, 9% were polycosmids.

The library was initially screened by assaying for β-ketothiolase activity. We had previously determined that this activity was easily assayable in *A. eutrophus* and that *E. coli* LE392 lysates (cleared of particulate matter) had undetectable levels of this enzyme. To facilitate screening, 5-ml cultures of each clone were grown and then pooled in groups of five for assay. Of the more than 200 pools that were screened, 6 were positive for β-ketothiolase activity (Table 1). Individual clones from each pool were screened, and the activity was traced to six clones. The activities of the β-ketothiolase-positive recombinants ranged between 50 and 15% of that found in *A. eutrophus* H16.

Further screening of β-ketothiolase-positive recombinants. Since many bacterial pathways are found either in operons or in clusters, we decided to assay for other activities of the biosynthetic portion of the pathway. The two most feasible assays were the acetoacetyl-CoA reductase assay and the assay for cellular PHB content. The assay for PHB synthetase, the third and final enzyme of the biosynthetic pathway, was not done because the assay requires a complex organic synthesis and purification and because the enzyme itself has been found to be very labile, even in its native system (7). Instead, the presence of the synthetase was inferred from the accumulation of its product.

Of the six recombinant clones analyzed, three (harboring pAE65, pAE175, and pAE689) were positive for acetoacetyl-CoA reductase activity and PHB production (Table 1). The clone harboring pAE65 expressed acetoacetyl-CoA reductase activity to a much higher level than did *A. eutrophus* H16 but produced a very small amount of PHB. On the other hand, acetoacetyl-CoA reductase activity in clones harboring pAE175 and pAE689 was extremely low when compared to that of *A. eutrophus* H16, but both clones produced PHB to approximately 50% of the concentration achieved in *A. eutrophus* H16. The reason for this apparent discrepancy between reductase levels and PHB production is not known, but it is possible that low reductase activities associated with high PHB production is the norm and that the pAE65 reductase activity is an artifact which results from scrambling of the DNA fragments in the cloning process. This interpretation is supported by the fact that restriction digest patterns of pAE175 and pAE689 were quite similar, whereas that of pAE65 had a limited likeness. We were not able to conduct Southern blot studies on pAE65 to confirm this

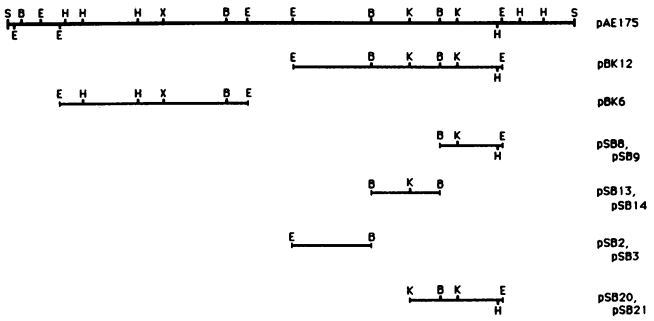

FIG. 1. Restriction endonuclease map of cosmid pAE175 insert showing subcloned restriction fragments. Abbreviations of restriction endonucleases are as follows: B, *Bgl*II; E, *Eco*RI; H, *Bam*HI; K, *Kpn*I; X, *Xho*I; S, *Sal*I.

possibility because pAE65 was lost upon subsequent passages of the culture.

Subcloning of pAE175 fragments. To further localize the pHB pathway on the cosmid DNA insert, pAE175 was mapped for restriction endonuclease sites, and two central *Eco*RI fragments were subcloned into pUC13 (Fig. 1). Two clones, representing the two fragments, were picked and analyzed for β-ketothiolase activity, acetoacetyl-CoA reductase activity, and PHB production (Table 2). Interestingly, high β-ketothiolase activity was detected in both clones. However, acetoacetyl-CoA reductase activity and PHB production were detected only in clones harboring pBK12 (14 kb in length). As in clones harboring pAE175 and pAE689, the acetoacetyl-CoA reductase activity in the clone harboring pBK12 was found in lower amounts than in *A. eutrophus*. PHB production in the pBK12-harboring clone was lower than that found in the PHB-producing cosmid clones.

The existence of two β-ketothiolase activities raises the possibility that the activity found on pBK12 is part of the biosynthetic portion of the PHB cycle and that the activity found on pBK6 is part of the catabolic portion of the pathway. If the pBK6 β-ketothiolase activity is catabolic, it could be proximal to the rest of the catabolic pathway. To test the possibility that pBK6 contained part or all of the biodegradative pathway, the clone was assayed for two of the remaining three catabolic enzymes, D-3-hydroxybutyrate dehydrogenase and succinyl-CoA transferase (20). Neither activity was found in lysates of *E. coli* harboring pBK6, *E. coli* harboring pBK12, or *E. coli* harboring pAE175, whereas both activities were easily measured in *A. eutrophus* H16 lysates (data not shown). Therefore, the β-ketothiolase activity encoded on pBK6 remains unexplained.

Plasmid pBK12 was further subcloned by digesting it with *Eco*RI and *Bgl*II. This resulted in two *Eco*RI-*Bgl*I fragments and one *Bgl*II fragment, all approximately the same size (4 kb). Six subclones, representing each portion of the pBK12 insert in duplicate, were picked and assayed for the three activities measured above. β-Ketothiolase activity and acetoacetyl-CoA reductase activity were detected in *E. coli* harboring plasmids pSB8 and pSB9, representing the rightmost *Bgl*II-*Eco*RI fragment, as depicted in Fig. 1. These activities were expressed at considerably higher levels than in *A. eutrophus*. Initially, it was thought that we had serendipitously placed the two genes under the control of the *lac* promoter just outside the multiple cloning site, because the specific activity of β-ketothiolase from these clones is similar to the value obtained from cloned *Zoogloea ramigera* β-ketothiolase under control of the *lac* promoter (16). However, we have since cloned the pathway in other small multicopy plasmids and have found similar, if not somewhat higher, levels of expression (data not shown).

The data from analyses of pSB8 and pSB9 were interpreted to mean that the first two enzymes of the PHB biosynthetic pathway are located on the 3,500-kb *Bgl*II-*Eco*RI fragment but that the third enzyme of the pathway, PHB synthetase, either was cleaved by *Bgl*II or is positioned to the left of the *Bgl*II site. In an effort to obtain the whole pathway on a sequence small enough to use in DNA sequence studies, a 5,200-base *Kpn*I-*Eco*RI fragment was cloned into pUC18, and two clones, harboring pSB20 and pSB21, were tested. When subjected to analysis, both clones containing this particular 5,200-base fragment exhibited β-ketothiolase and acetoacetyl-CoA reductase activities and produced PHB. The PHB accumulation was particularly striking, with the subclones accumulating nearly as much, or more, PHB as *A. eutrophus* H16 (Table 2).

Comparison of *A. eutrophus* H16 DNA with cloned DNA. Because the manner in which the PHB pathway was cloned left open the possibility that the cloned fragment was a product of scrambling, Southern blot analysis was used to demonstrate that the PHB biosynthetic pathway in *A. eutrophus* H16 has the same restriction pattern as that of the cloned PHB DNA. Digested pAE175 was compared to

TABLE 2. Analysis of subclones for enzyme activity and PHB production

Bacterium[a]	Plasmid	β-Ketothiolase activity[b]	Acetoacetyl-CoA reductase activity[c]	mg of PHB/ml of culture	% PHB
E. coli LE392	None	0.0	0.0	0.0	0
A. eutrophus H16	None	3.5	11.2	1.64	49
E. coli LE392	pAE175	1.7	1.2	0.71	19
E. coli DH5	pBK6	1.6	0.0	0.0	0
E. coli DH5	pBK12	2.0	4.5	0.71	18
E. coli DH5	pSB2	0.0	0.0	0.0	0
E. coli DH5	pSB3	0.0	0.0	0.0	0
E. coli DH5	pSB8	59.2	50.1	0.0	0
E. coli DH5	pSB9	20.2	8.7	0.0	0
E. coli DH5	pSB13	0.0	0.0	0.0	0
E. coli DH5	pSB14	0.0	0.0	0.0	0
E. coli DH5	pSB20	2.7	0.7	2.82	54
E. coli DH5	pSB21	2.4	0.6	2.28	39

[a] For enzyme assays, bacteria were grown in LB. For the PHB assay, bacteria were grown in LB plus 1% gluconate.
[b] Micromoles of acetoacetyl-CoA degraded per minute per milligram of protein.
[c] Micromoles of NADPH reduced per minute per milligram of protein.

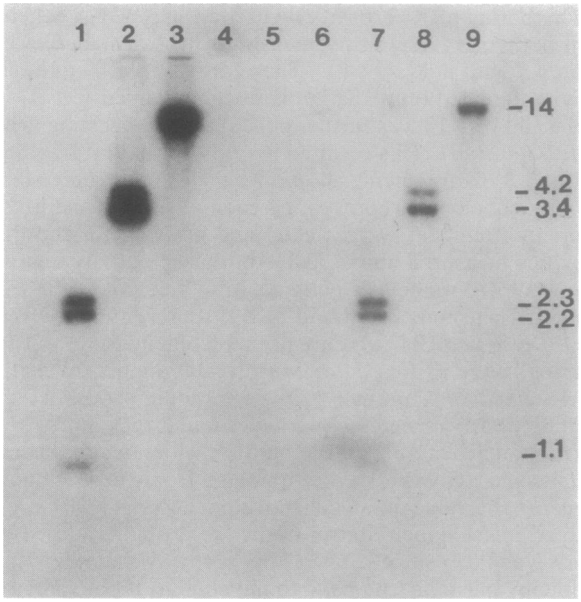

FIG. 2. Southern blot analysis of DNA from *E. coli* harboring pAE175 (lanes 1 to 3) and of *E. coli* LE392 (lanes 4 to 6) and *A. eutrophus* H16 genomic DNA (lanes 7 to 9). Lanes 1, 4, and 7, *Eco*RI; lanes 2, 5, and 8, *Eco*RI-*Bgl*II; lanes 3, 6, and 9, *Sal*I.

digests of DNA extracted from *A. eutrophus* H16 and *E. coli* LE392. Restriction enzymes used were *Eco*RI, *Eco*RI-*Bgl*II, and *Sal*I, respectively. A gel-purified 5.2-kb PHB fragment was labeled and used as a probe. The blot reveals that the PHB biosynthetic pathway is located on a 14-kb *Eco*RI fragment in *A. eutrophus* H16 (Fig. 2, lane 7) and in pAE175 (Fig. 2, lane 1). No hybridization could be detected to any DNA fragments from *E. coli* LE392 (Fig. 2, lanes 4 to 6). Further digests of pAE175 and *A. eutrophus* genomic DNA manifested the same restriction patterns, indicating that the cloned PHB biosynthetic pathway was the same as that found in *A. eutrophus* H16.

PHB in *E. coli*. The high levels of PHB that were detected in *E. coli* suggested that the PHB could be seen by use of a microscope, particularly if it were produced in granules. To test this possibility, 24-h cultures of *A. eutrophus* H16 and of *E. coli* harboring pSB20 were stained for 15 s with crystal violet. The crystal violet is taken up by the cells, but PHB granules are refractile to the stain. Under the oil immersion lens, PHB granules in *A. eutrophus* were evident as fuzzy nonstaining areas between stained regions of the bacterium (Fig. 3A). Granules in *E. coli* were much more distinct (Fig. 3B). Granule formation in *E. coli* appears to differ from that in *A. eutrophus* H16 in that the granules in *E. coli* were more numerous and were often larger in diameter than the cell. PHB granules in *A. eutrophus* H16 did not usually distend the cell membrane.

To corroborate the PHB assays and demonstrate possible differences between PHB produced in its native state and PHB produced in the recombinants, PHB was extracted from bacterial cells and subjected to IR analysis (22). The results demonstrate that the IR spectra of PHB from *A. eutrophus* H16, *E. coli* harboring pAE175, and *E. coli* harboring pSB20 are virtually identical (Fig. 4). In addition, the PHB spectra were very similar to those from other organisms (5, 22).

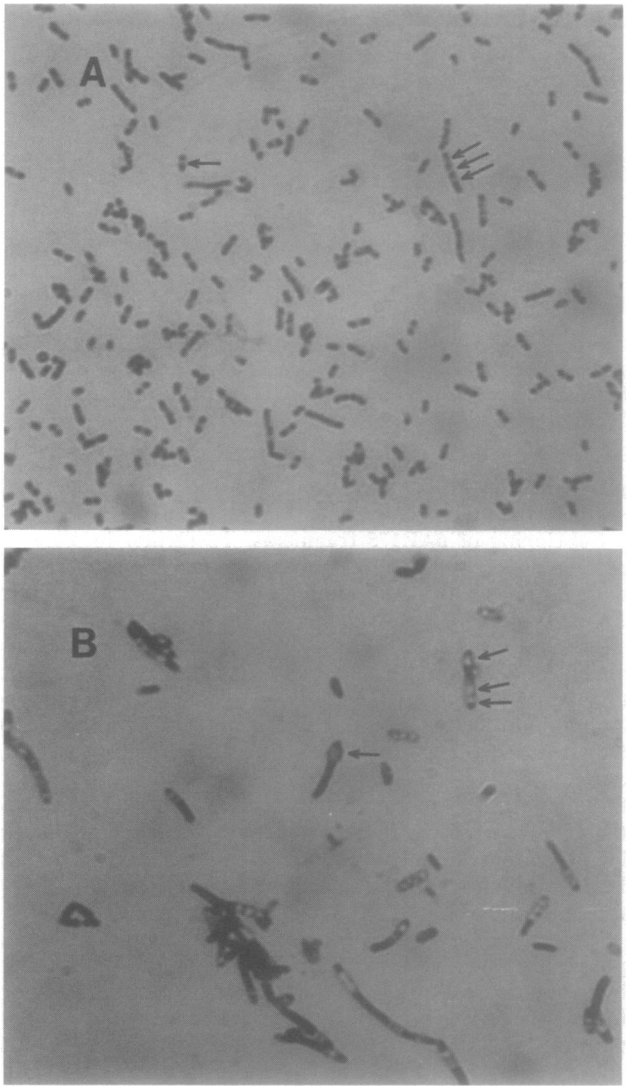

FIG. 3. Photomicrograph of *A. eutrophus* H16 (A) and of *E. coli* harboring pSB20 (B) showing intracellular granules (arrows). Magnification, ca. ×3,000.

DISCUSSION

Alterations in environmental conditions often cause dramatic shifts in intermediary metabolism. Many of these shifts are controlled by global regulatory networks capable of coordinated induction or repression of many enzymes. Examples include sigma factors produced during *Bacillus* sporulation (12), the cyclic AMP/cyclic AMP receptor protein system in *E. coli* carbon-source shifts (6), and the HtpR signal polypeptide required for the *E. coli* heat shock response (14).

PHB synthesis is induced in *A. eutrophus* H16 and in other organisms by many of the same conditions that induce these regulons. Indeed, the role of PHB as a carbon and energy sink is central to many other cellular processes during periods of environmental limitation and strongly suggests regulon control. Therefore, we are studying PHB as a model for coordinated regulation of stress-induced metabolism and have presented here an initial genetic analysis of the *A. eutrophus* H16 PHB biosynthetic pathway.

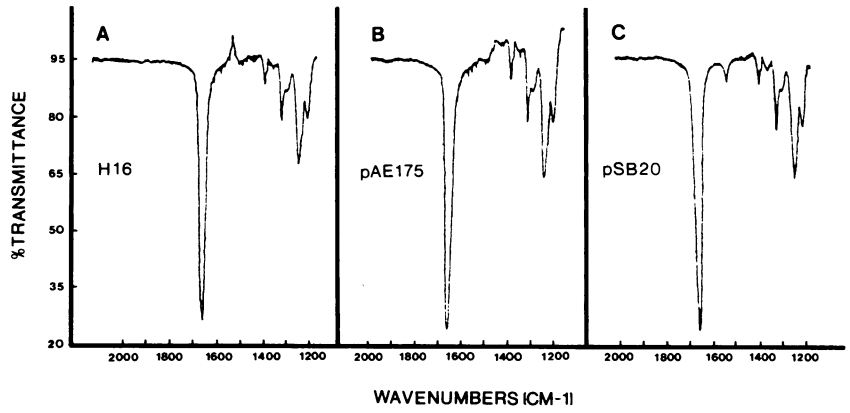

FIG. 4. IR spectra of PHB extracted from *A. eutrophus* (A), *E. coli* harboring pAE175 (B), and *E. coli* harboring pSB20 (C).

Although much information is available concerning the regulation of the PHB pathway, very little is known about the molecular genetics of PHB metabolism. In this paper, we have reported the cloning of the PHB biosynthetic pathway by use of cosmids and several different plasmid vectors. Most clones exhibit substantial β-ketothiolase activity and PHB production in comparison to *A. eutrophus* H16. Both pAE175 and pAE689 produced PHB to approximately 50% of the level achieved in *A. eutrophus* H16 while expressing reductase levels that were less than 2% of reductase levels in *A. eutrophus* H16.

In subcloning experiments, we have identified two different β–ketothiolase-positive subclones. This result could be explained by the presence of two functionally different β-ketothiolases. That is, there may be a biosynthetic thiolase and a catabolic thiolase. However, if the β-ketothiolase activity encoded by pBK6 is indeed due to a catabolic enzyme, the gene is evidently located apart from the rest of the genes encoding catabolic PHB enzymes or is located on the very edge of the DNA fragment with the other catabolic enzymes being encoded on an adjacent DNA fragment. Another possibility is that the two genes result from a gene duplication. Andersen and Wilke-Douglas have documented the existence of duplicate genes in *A. eutrophus* (1). However, in this instance these do not appear to be duplicate genes because in Southern blot studies the PHB probe did not bind to the 12-kb *Eco*RI fragment on which the "alternate" β-ketothiolase activity is encoded.

We were struck by the levels of intracellular PHB that were accumulated in *E. coli*. These levels approached 90% of the bacterial cell dry weight in some subclones, and PHB was observable as large intracellular bodies. Because the two bacterial genera are somewhat related and because the expression of *A. eutrophus* genes in *E. coli* has been documented (1), we were not surprised that the pathway was expressed to easily measurable levels (particularly in light of a possible gene dosage effect due to cloning into multicopy plasmids). Nevertheless, to obtain such high levels of expression implies either a high degree of transcriptional versatility or a high degree of transcriptional homology. The versatility of the pathway is certainly corroborated by the existence of this apparently nonessential pathway in so many bacterial genera.

The presence of granules that are likely to be composed of PHB was noted. We do not know whether these granules represent inclusion bodies or true PHB granules. This suggests that, if the granules are indeed true PHB granules, whose production is encoded by genes on a 5.2-kb DNA fragment, granule formation does not require the manufacture of extensive cellular machinery or utilizes existing cellular structures and functions.

These clones represent an excellent opportunity for us to determine the relationship between PHB transcriptional control and transcriptional control in other regulons. Preliminary experiments lead us to believe that the PHB biosynthetic pathway in *E. coli* is controlled in a manner similar to that found in *A. eutrophus* H16 (nitrogen and oxygen limitation). In future experiments, we will attempt to determine the mode of this control and the genetic sequences operational in this control.

ACKNOWLEDGMENTS

We thank the following undergraduate researchers who participated in this work: Mehrdad Ebadi-Tehrani, Scott Hamilton, Leslie Metker, Becky Johnson, Stacey Shults, Jeff Hollar, and Tracy Thranhardt. We also thank Wendy Renfroe for her excellent technical assistance.

This work was supported by Jeffress Memorial Trust Grant no. J-84 and by grant no. DMB-8605843 from the National Science Foundation.

LITERATURE CITED

1. **Andersen, K., and M. Wilke-Douglas.** 1984. Construction and use of a gene bank of *Alcaligenes eutrophus* in the analysis of ribulose bisphosphate carboxylase genes. J. Bacteriol. **159**:973–978.
2. **Burns, D. M., and I. R. Beacham.** 1983. A method for the ligation of DNA following isolation from low melting temperature agarose. Anal. Biochem. **135**:48–51.
3. **Carter, I. S., and E. A. Dawes.** 1979. Effect of oxygen concentration and growth rate on glucose metabolism, poly-β-hydroxybutyrate biosynthesis and respiration of *Azotobacter beijerinckii*. J. Gen. Microbiol. **110**:393–400.
4. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **14**:135–266.
5. **Fernandez-Castillo, R., F. Rodriguez-Valera, J. Gonzalez-Ramos, and F. Ruiz-Berraquero.** 1986. Accumulation of poly(β-hydroxybutyrate) by halobacteria. Appl. Environ. Microbiol. **51**:214–216.
6. **Gottesman, S.** 1984. Bacterial regulation: global regulatory networks. Annu. Rev. Genet. **18**:415–441.
7. **Griebel, R. J., and J. M. Merrick.** 1971. Metabolism of poly-β-hydroxybutyrate: effect of mild alkaline extraction on native poly-β-hydroxybutyrate granules. J. Bacteriol. **108**:782–789.
8. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.

9. **Heinzle, E., and R. M. Lafferty.** 1980. A kinetic model for growth and synthesis of poly-β-hydroxybutyric acid (PHB) in *Alcaligenes eutrophus* H16. Eur. J. Appl. Microbiol. Biotechnol. **11**:8–16.
10. **Jackson, F. A., and E. A. Dawes.** 1976. Regulation of the tricarboxylic acid cycle and poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinkii* grown under nitrogen or oxygen limitation. J. Gen. Microbiol. **97**:303–312.
11. **Lemoigne, M.** 1926. Origine de l'acide β-oxo-butyrique obtenue par autolyse microbienne. C.R. Soc. Biol. **95**:1359–1360.
12. **Losick, R., and J. Pero.** 1981. Cascades of sigma factors. Cell **25**:582–584.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Neidhart, F. C., R. A. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18**:295–329.
15. **Oeding, V., and H. G. Schlegel.** 1973. β-Ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-β-hydroxybutyrate metabolism. Biochem. J. **134**:239–248.
16. **Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey.** 1987. Biosynthetic thiolase from *Zoogloea ramigera*. III. Isolation and characterization of the structural gene. J. Biol. Chem. **262**:97–102.
17. **Pritchard, R. H., and I. B. Holland.** 1985. Basic cloning techniques: a manual of experimental procedures. Blackwell Scientific Publications, London.
18. **Repaske, R., and A. C. Repaske.** 1976. Quantitative requirements for exponential growth of *Alcaligenes eutrophus*. Appl. Environ. Microbiol. **32**:585–591.
19. **Senior, P. J., and E. A. Dawes.** 1971. Poly-β-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in *Azotobacter beijerinkii*. Biochem. J. **125**:55–68.
20. **Senior, P. J., and E. A. Dawes.** 1973. The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinkii*. Biochem. J. **134**:225–238.
21. **Sonnleitner, B., E. Heinzle, G. Braunegg, and R. M. Lafferty.** 1979. Formal kinetics of poly-β-hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus* H 16 and *Mycoplana rubra* R 14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures. Eur. J. Appl. Microbiol. **7**:1–10.
22. **Wakisaka, Y., E. Masaki, and Y. Nishimoto.** 1982. Formation of crystalline g-endotoxin or poly-β-hydroxybutyric acid granules by asporogenous mutants of *Bacillus thuringiensis*. Appl. Environ. Microbiol. **43**:1473–1480.
23. **Ward, A. C., and E. A. Dawes.** 1973. A disk assay for poly-β-hydroxybutyrate. Anal. Biochem. **52**:607–613.
24. **Ward, A. C., B. I. Rowley, and E. A. Dawes.** 1977. Effect of oxygen and nitrogen limitation on poly-β-hydroxybutyrate biosynthesis in ammonium-grown *Azotobacter beijerinkii*. J. Gen. Microbiol. **102**:61–68.