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Molecular Analysis of the *Alcaligenes eutrophus* Poly(3-Hydroxybutyrate) Biosynthetic Operon: Identification of the N Terminus of Poly(3-Hydroxybutyrate) Synthase and Identification of the Promoter

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Molecular methods have been applied to analyze the expression of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) (PHB) synthase gene (*phbC*). The translational initiation codon was identified by analysis of the amino acid sequence of a PHB synthase–β-galactosidase fusion protein. This protein was purified to almost gel electrophoretic homogeneity by chromatography on DEAE-Sephacel and on aminophenyl-β-D-thiogalactopyranoside–Sepharose from cells of *A. eutrophus* which harbored a *phbC'-'lacZ* fusion gene. A sequence (TTGACA-18N-AACAAT), exhibiting striking homology to the *Escherichia coli* σ^{70} promoter consensus sequence, was identified approximately 310 bp 5′ upstream from the translation initiation codon. An S1 nuclease protection assay mapped the transcription start point of *phbC* 6 bp downstream from this promoter. The location of the promoter was confirmed by analyzing the expression of active PHB synthase in clones of *E. coli* harboring 5′ upstream deletions of *phbC* ligated to the promoter of the *lacZ* gene (*lacZp*) in a Bluescript vector. Plasmids do181 and do218, which were deleted for the first 108 or 300 bp of the *phbC* structural gene, respectively, conferred the ability to synthesize large amounts of different truncated PHB synthase proteins to the cells. These proteins contributed to approximately 10% of the total cellular protein as estimated from sodium dodecyl sulfate-polyacrylamide gels. The modified PHB synthase encoded by plasmid do181 was still active. Clones in which the *lacZp-'phbC* fusion harbored the complete *phbC* structural gene plus the *phbC* ribosome binding site did not overexpress PHB synthase.

In *Alcaligenes eutrophus*, synthesis of poly(3-hydroxybutyric acid) (PHB) starts from acetyl coenzyme A (acetyl-CoA) and is accomplished by three enzymatic reactions: (i) condensation of two acetyl-CoA units to acetoacetyl-CoA, (ii) chiral reduction of acetoacetyl-CoA to D-(−)-3-hydroxybutyryl-CoA, and (iii) polymerization of D-(−)-3-hydroxybutyrate units. The *A. eutrophus* PHB-biosynthetic genes *phbA* (for 3-ketothiolase), *phbB* (NADPH-dependent acetoacetyl-CoA reductase), and *phbC* (PHB synthase) have been cloned recently (40, 42). The genes are clustered and are presumably organized in one operon. The genes were expressed in *Escherichia coli* and in different species of the genus *Pseudomonas* belonging to rRNA homology group I; they conferred the ability to accumulate polyesters consisting of 3-hydroxybutyrate on most of the recombinant cells (40, 42–45).

Subcloning experiments (28, 29, 41, 42), analysis of transposon Tn*5*-induced PHB-negative mutants (40, 41), and nucleotide sequence data (28, 29; this study) provided evidence that the three genes are organized in one operon (*phbC-phbA-phbB*). It turned out that the sequence data obtained in our and in Dr. Sinskey's laboratory were almost identical. We hesitated to publish this sequence because the N-terminal amino acid sequence of the PHB synthase protein was not known at that time and the promoter of *phbC* had not been mapped. Although the nucleotide sequence of an open reading frame for *phbC* was published recently, the transcription and translational initiation sites were not mapped (29). Furthermore, the molecular mass of the gene product most likely representing PHB synthase protein obtained in maxicell experiments was only 58,000 Da, whereas the molecular mass of PHB synthase predicted from the amino acid sequence deduced from the putative open reading frame was 63,940 Da (29). This indicated that other translational initiation codons for *phbC* were possible within the detected open reading frame.

Optimum expression of the *A. eutrophus* PHB-synthetic pathway in other bacteria or even in plants (31), which would provide greater flexibility for the production of polyhydroxyalkanoates, will require exact knowledge not only of the properties of the enzymes (15–17) involved in the synthesis of PHB, but also of the structures which regulate the expression of the corresponding genes. In the present study we analyzed various *phbC'-'lacZ* and *lacZ'-phbC'* fusions and determined the translation as well as the transcription start sites of *phbC*.

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MATERIALS AND METHODS

Bacterial strains and media. The strains of *A. eutrophus* and of *E. coli* and the plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium (25), whereas *A. eutrophus* was grown in mineral salts medium (MM) (39) supplemented with 0.5% (wt/vol) gluconate or fructose.

DNA manipulations. Plasmid DNA was isolated from crude lysates by the alkaline extraction procedure (5, 25).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
A. eutrophus		
H16	Wild type	DSM[b]428
HF09	Hox$^-$ Hno$^-$ acetoin$^-$	10, 38
HF149	Hox$^-$ Nit$^-$ acetoin$^-$ Km^r, Tn*5*-induced mutant	20
H1070	Hox$^-$ Nit$^-$ acetoin$^-$ Km^r, Tn*5*-induced mutant	11
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi relA1 lacZ*Δ*M15 lacQ* F′ *proAB* Tn*10*	7
ΔMN5-25–ΔMN5-3181	Derivative strains of ΔMN5 which harbor different *phbC′-′lacZ* fusions (for details, see text)	8, 32, 35
Plasmids		
Bluescript KS−	Ap^r, *lacPOZ′*	Stratagene, San Diego, Calif.
Bluescript SK−	Ap^r, *lacPOZ′*	Stratagene, San Diego, Calif.
pBP42	Derivative of pMC1403 harboring a *phbC′* gene	32
pPL76	Derivative of pVK101 harboring a *phbC′-′lacZ* fusion gene	32
pSAP33	Bluescript $\overrightarrow{KS}-::\overrightarrow{SA}35$	41
pSAP37	Bluescript $\overrightarrow{KS}-::\overleftarrow{SA}35$	41
pSK2665	Bluescript $\overrightarrow{KS}-::\overleftarrow{SE}52$	41
do95, do181, do186, do218, do238, do295	Derivatives of pSAP33 harboring deletions in *phbC* or 5′ upstream region (for details, see text)	This study
pSK2484	pUC9-1::$\overrightarrow{SE}52$	This study
pSK3958	pUC9-1::$\overrightarrow{SE}52$ (Δ*Kpn*I)	This study

[a] Symbols: → →, PHB-biosynthetic genes oriented in direction of *lacPOZ′*; → ←, PHB-biosynthetic genes oriented in direction opposite that of *lacPOZ′*.
[b] DSM, Deutsche Sammlung von Mikroorganismen.

Isolated plasmid DNA was digested with various restriction endonucleases under the conditions described by Maniatis et al. (25) or by the manufacturer. DNA restriction fragments were isolated from agarose gels by using the Geneclean kit (48). All other DNA-manipulating enzymes were used as described by the manufacturer.

Transformation. For transformation, *E. coli* was cultivated in LB medium containing 20 mM each of $MgCl_2$ and $MgSO_4$ (13) at 37°C. Competent cells were prepared and transformed by the calcium chloride procedure (25).

DNA sequence analysis. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (36) with alkaline denatured double-stranded plasmid DNA (9) and with [α-^{35}S]dATP by using a T7 polymerase kit according to the manufacturer's protocol.

Preparation of RNA. Total RNA was isolated from *A. eutrophus* by the hot phenol-chloroform procedure described by Oelmüller et al. (27).

Determination of transcription start site. For the determination of the transcription start site, a nuclease protection assay was used. The hybridization conditions were as described in detail by Berk and Sharp (4) and Maniatis et al. (25), and the S1 nuclease reactions were conducted by the method described by Aldea et al. (1). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with pBL42 DNA as a template. For the annealing reaction, an oligonucleotide (5′-TCGGTCA AGGTCCCGTC-3′), which was complementary to positions 691 to 707 (downstream from the *Sma*I site), was used for ^{35}S labeling. For all mapping experiments, 40 μg of RNA was mixed with the labeled DNA fragment; the specific labeling rate was higher than 10^7 cpm/μg of DNA.

Preparation of crude extracts. Approximately 6 g (wet weight) of cells of *A. eutrophus* H16 were suspended in 12 ml of buffer A (50 mM Tris hydrochloride [pH 7.4], 10 mM mercaptoethanol, 10 mM magnesium chloride, 10 mM EDTA), which was supplemented with 200 μg of phenylmethylsulfonyl fluoride per ml and 10 μg of DNase I per ml. The cells were disrupted by three passages through a French press at 96 MPa. Crude cellular extracts and the soluble protein fractions from cells of *E. coli* were obtained by ultrasonic disintegration as described recently (40).

Enzyme assays. Activities of 3-ketothiolase (EC 2.3.1.9) and NADPH-dependent acetoacetyl-CoA reductase (EC 1.1.1.36) were determined by UV spectroscopic assays by the methods described by Nishimura et al. (26) and Lynen and Wieland (24), respectively. PHB synthase activity was measured by a radiometric assay with 3-[^{3}H]hydroxybutyryl-CoA as described recently (40). β-Galactosidase activity was determined as described by Platt et al. (30).

Protein determination. The protein content in samples was determined as described by Beisenherz et al. (3). To sediment interfering material, the samples were centrifuged for 30 min at 3,000 × *g* in a bench centrifuge prior to the determination of the absorbance.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)- and mercaptoethanol-denatured proteins were separated in 11.5% (wt/vol) polyacrylamide gels in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 0.1% [wt/vol] SDS). The size of the gel was 1.5 by 140 by 140 mm, and electrophoresis was done for 2.5 h at 4°C and at a constant 40 mA (23). Proteins were stained with Coomassie brilliant blue (49) or by the silver stain procedure (18). Standard proteins from commercially available molecular weight marker kits were used as references.

Determination of PHB. To determine the PHB content, the polymer of 4 ± 1 mg of freeze-dried cells was transformed to the constituent 3-hydroxycarboxylic acid methyl esters by methanolysis (6). These esters were analyzed by gas chromatography as described recently in detail (6, 44).

Isolation of the *phbC′-′lacZ* fusion protein. After phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM to the crude extract obtained from 6 g (wet weight) of cells, cellular debris was removed by centrifugation in an

RC-5B centrifuge (Du Pont de Nemours GmbH, Bad Homburg, Federal Republic of Germany [FRG]) for 20 min at 13,000 × *g*. Then, 24 ml of the supernatant was applied to a DEAE-Sephacel column (2.6 by 8 cm, 40-ml bed volume [BV]). The column was subsequently washed with 2 BV of buffer A, 6 BV of buffer B (buffer A plus 150 mM NaCl), and 4 BV of buffer C (buffer A plus 400 mM NaCl). The protein solution, which was eluted with buffer C, was adjusted to 1.6 M NaCl (47) and applied to a column of *p*-aminophenyl-β-D-thiogalactopyranoside (APTG)–Sepharose (1.6 by 5 cm, 10-ml BV) (2), which was equilibrated with buffer D (buffer A plus 1.6 M NaCl). This column was washed with 300 ml of buffer D, and proteins were eluted with 100 mM sodium borate, pH 10.0, which contained 10 mM mercaptoethanol. In order to prevent alkaline denaturation of the proteins, the eluate was collected in 0.3 volume of 1 M Tris hydrochloride, pH 6.8. Fractions containing β-galactosidase activity were combined, and the proteins were concentrated to approx. 3.5 mg/ml in an ultrafiltration cell (Amicon, Witten/Ruhr, FRG) with a YM30 membrane and by acetone precipitation.

N-terminal sequence analysis. The sequence analysis was performed with a model 477A pulsed liquid phase peptide-peptide sequencer (19) and a model 120A on-line phenolthiohydrantoin amino acid analyzer (33) according to the instructions of the manufacturer (Applied Biosystems, Weiterstadt, FRG).

Chemicals and reagents. Acetyl-CoA, acetoacetyl-CoA, 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and agarose type NA were obtained from Sigma Chemie (Deisenhofen, FRG). Restriction endonucleases, T4 ligase, T4 DNA polymerase, and S1 nuclease were obtained from GIBCO/Bethesda Research Laboratories GmbH (Eggenstein, FRG). IPTG (isopropyl-β-D-thiogalactopyranoside) was obtained from BIOMOL Feinchemikalien GmbH (Hamburg, FRG). Calf intestinal alkaline phosphatase was purchased from C. F. Boehringer & Soehne (Mannheim, FRG). The exonuclease III/mung bean nuclease kit was obtained from Stratagene Cloning Systems (La Jolla, Calif.), and the Geneclean kit was from BIO 101 Inc. (La Jolla, Calif.); the T7 sequencing kit, RNase-free DNase, DEAE-Sephacel, and molecular weight calibration kits were from Pharmacia (Uppsala, Sweden). Sodium [^{3}H]borohydride and [α-^{35}S]dATP were purchased from Amersham Buchler GmbH & Co. KG (Braunschweig, FRG).

RESULTS

Subcloning of the PHB-biosynthetic genes. Subcloning of the 12.5-kb *Eco*RI restriction fragment PP1, which was recently isolated from *A. eutrophus* genomic DNA and which conferred the ability to accumulate PHB on *E. coli* and other bacteria (40, 43), revealed that plasmid pSK2665, which harbors the 5.2-kb *Sma*I-*Eco*RI subfragment SE52, harbored all three genes needed for synthesis of PHB from acetyl-CoA. In addition, synthesis of active PHB synthase was also accomplished by plasmids pSAP33 and pSAP37, which harbored the 3.5-kb *Sma*I-*Apa*I subfragment SA35 of SE52, although the activity was much lower than with plasmids harboring SE52. Cells of *E. coli* which harbored plasmid pSK3958 did not synthesize active PHB synthase, whereas expression of NADPH-dependent acetoacetyl-CoA reductase and of β-ketothiolase was not affected. In this plasmid, the 37-bp *Kpn*I fragment within the open reading frame for *phbC* has been deleted by subsequent treatment of pSK2484 with *Kpn*I and T4 ligase. Neither pSK3958, pSAP33, nor pSAP37 conferred the ability to synthesize and accumulate PHB on *E. coli*.

Determination of the nucleotide sequence of *phbC*. When fragment SA35 was ligated to the Bluescript vector KS−, two hybrid plasmids, pSAP33 and pSAP37, were isolated which harbored SA35 in one or the other direction. To obtain unidirectional nested deletions, both plasmids were linearized by treatment with *Apa*I and *Eco*RI. Subsequently, the DNA was treated with exonuclease III, and overhanging single-stranded DNA was removed with mung bean nuclease. Deleted plasmids were isolated in *E. coli* XL1-Blue. The nucleotide sequence of SA35 was obtained from overlapping partial sequences determined throughout for both strands by the dideoxy chain-termination method and by employing universal primers. The nucleotide sequence was identical with the sequence published recently (29) except for position 281; at that position, an additional G was detected in our laboratory. As the sequence has already been published (28, 29), only the region which is relevant for further experiments is shown in Fig. 1.

Analysis of *phbC'-'lacZ* gene fusions. The 1,767-bp open reading frame, which started 842 bp downstream from the *Sma*I site and which most likely harbored the *A. eutrophus* structural gene for PHB synthase, was preceded by a tentative ribosome binding site. That *phbC* was located within this open reading frame was assumed from the results of subcloning experiments (29, 41; this study) and from molecular as well as enzymatic characterization of Tn*5*-induced mutations (40). Peoples and Sinskey indicated a certain degree of homology in the deduced amino acid sequences of the synthases from *A. eutrophus* and *Pseudomonas oleovorans* (29). This would support the previous results. However, the exact translation start site has not been experimentally proven, and data for the N-terminal amino acid sequence of the PHB synthase were not provided.

Various clones of *E. coli* ΔMN5, which harbored hybrid plasmids consisting of *Bal*31-treated derivatives of the 4.2-kb *Bgl*II restriction fragment of PP1 (40, 41) ligated to the promoter probe vector pMC1403 (32), were analyzed in order to localize the fusions. Sequence analysis with an oligonucleotide which hybridized to the *lacZ'* gene in the outward direction as the primer revealed that the distances between these fusion sites (Fig. 1) and the first nucleotide of the open reading frame encoding *phbC* were multiples of three in all 15 clones forming blue colonies (strains ΔMN5-3166, -3167, -3168, -3169, -3170, -3171, -3172, -3173, -3174, -3175, -3176, -3177, -3179, -3180, and -3181). In contrast, the fusions of clones forming white colonies were either not in frame (strains ΔMN5-25, -35, -38, and -39, Fig. 1) or occurred in the opposite direction (strains ΔMN5-31 and ΔMN5-37, not shown). These results confirmed the hypothesis that the promoter of the *A. eutrophus* PHB-biosynthetic operon was most likely located on this *Bgl*II restriction fragment. The results confirmed our strategy to clone this region upstream of a promoterless *lacZ'* gene in order to get expression of β-galactosidase in *A. eutrophus* (see plasmid pPL76 in reference 32).

In order to determine the N-terminus of the PHB synthase and to identify the translational initiation codon of *phbC* within the open reading frame, the *phbC'-'lacZ* gene fusion of the plasmid harbored by clone ΔMN5-3166 was ligated to the broad-host-range vector pVK101 (32) and transferred into *A. eutrophus* H16 by conjugation. This ensured that the fusion gene was expressed in the natural host of *phbC*. The fusion protein was purified from crude extracts obtained from gluconate-grown cells of a transconjugant, which were

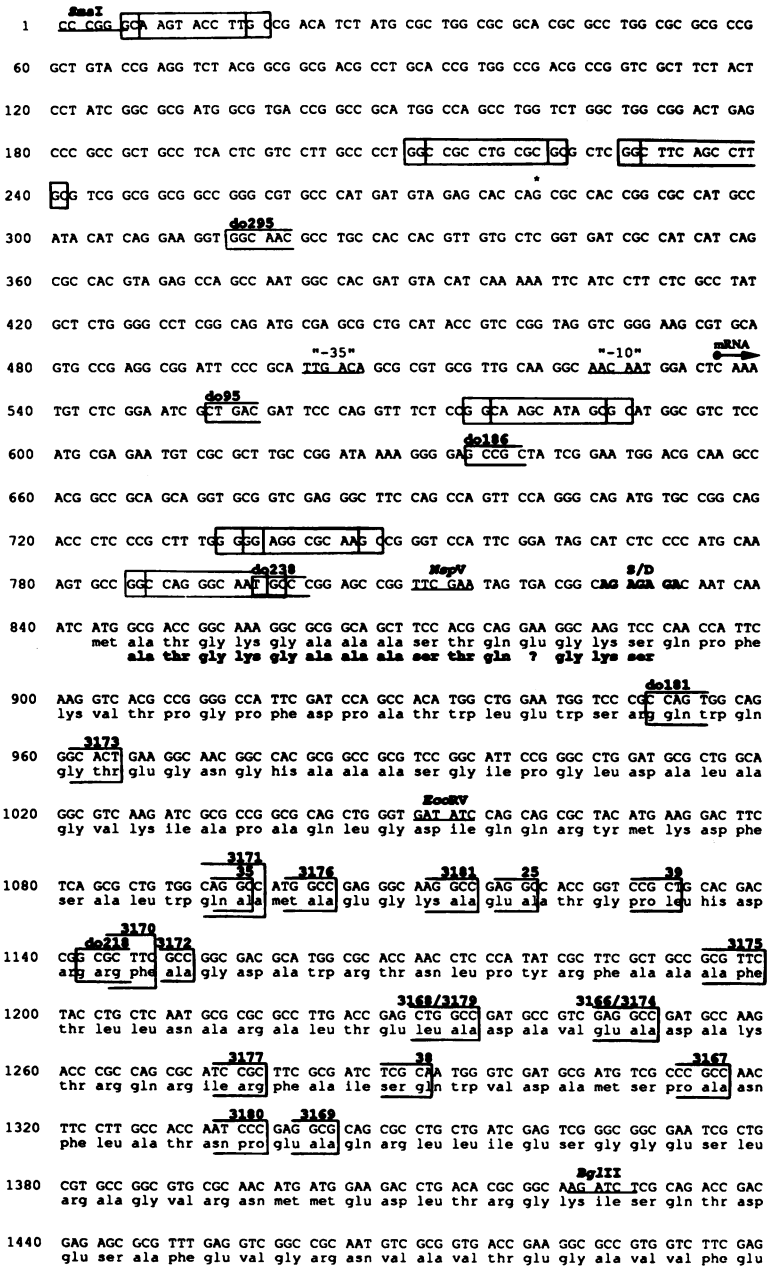

FIG. 1. Nucleotide sequence of the upper part of *phbC* and its 5′ upstream region and gene fusion products. The potential ribosome binding site (S/D) is shown in boldface letters. The putative promoter is underlined, and a dot indicates the transcription start site as determined by the S1 nuclease protection assay. Conserved nucleotides of potential σ^{54} promoters are boxed. The derived amino acid sequence is given in the three-letter code. The amino acid sequence determined for the N-terminus of the *phbC′-′lacZ* fusion gene product is also given in the three-letter code but in boldface letters. An asterisk at position 281 indicates the only deviation to the sequence published recently (29). Two- or four-digit numbers indicate fusions of the *Bal*31-treated 4.2-kb *Bgl*II restriction fragment of PP1 with the promoter probe vector pMC1403. Deleted derivatives of plasmid pSAP33 are designated with do and a two- or three-digit number. Those parts of the fusion genes which contain *A. eutrophus* genomic DNA are boxed, with the foreign DNA located outside the box.

cultivated under conditions allowing accumulation of PHB. Subsequent anion-exchange chromatography on DEAE-Sephacel and affinity chromatography on APTG-Sepharose yielded a nearly electrophoretically homogeneous protein preparation.

The proteins were blotted from an SDS-polyacrylamide gel onto a PVDF membrane. The area of the membrane, which represented a protein with a molecular weight of approximately 120,000, was cut off and used for amino acid sequence analysis. The N-terminal 15 amino acids of the fusion protein were determined (NH_2-Ala-Thr-Gly-Lys-Gly-Ala-Ala-Ala-Ser-Thr-Gln-?-Gly-Lys-Ser). Only at position 12 was no clear signal obtained; glutamine and threonine were the candidates for this position. The sequence was identical to the amino acid sequence deduced for the region located 846 to 890 bp downstream from the *Sma*I restriction

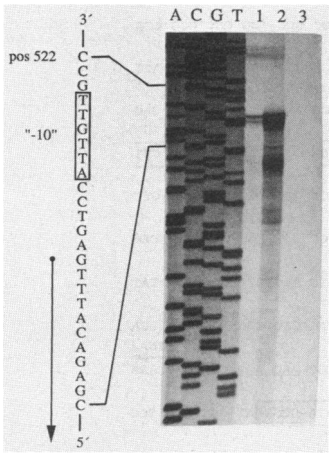

FIG. 2. S1 nuclease protection assay of the *phbC* transcript. Lanes ACGT, Standard sequencing reactions to size the mapping signals; lane 1, *A. eutrophus* H16; lane 2, *A. eutrophus* H16(pPL76); lane 3, control without RNA. The −10 region is shown. The arrow indicates the origin and direction of transcription. pos, Position.

site (Fig. 1). As alanine was the N-terminal residue of the analyzed protein, the methionine residue was most likely removed by posttranslational modification; the removal of methionine is consistent with the observation made for proteins in *E. coli* (37). Therefore, the PHB synthase structural gene starts definitely 843 bp downstream from the *Sma*I site at the beginning of the open reading frame, as proposed by Peoples and Sinskey (29).

Identification of the promoter. The region which exhibited the highest degree of homology to the *E. coli* σ^{70} promoter consensus sequence was located approximately 300 bp upstream of the translational initiation codon (Fig. 1). In order to localize the position of the promoter, *A. eutrophus* H16 and H16 harboring plasmid pPL76 were cultivated under nitrogen-limited conditions (MM with 0.05% [wt/vol] ammonium chloride), with gluconate as the sole carbon source. Total RNA was isolated from PHB-accumulating cells, which were harvested in the stationary growth phase. An S1 nuclease protection assay revealed that both strains transcribed the PHB synthase from the C located 6 bp downstream from the putative −10 region (Fig. 1 and 2). That the signal was much stronger with RNA prepared from the recombinant strain harboring pPL76 is probably the result of the gene dosage effect resulting from the presence of several copies of *phbC'* in the cells.

It should be mentioned that several regions which were identical to the *E. coli* σ^{54} consensus sequence (GG-10N-GC) are located upstream of *phbC*. These potential *rpoN*-dependent promoter structures were located with a spacing of 43, 95, 254, 584, 601, and 824 nucleotides upstream of *phbC*. The pleiotropic mutant strains HF09, HF149, and H1070 are defective in the synthesis of an alternative sigma factor of *A. eutrophus*, which is homologous to σ^{54} of enterobacteria (34). However, these mutants are not altered with respect to the ability to accumulate PHB (not shown in detail); therefore, the regions probably do not represent active promoters.

Analysis of clones deleted in the promoter region or in the N-terminal region of PHB synthase. In six derivative plasmids of pSAP33 harboring nested deletions originating from the *Sma*I site generated by the exonuclease III-mung bean nuclease system, deletions were mapped by determining the nucleotide sequence of the *lacZp'-'phbC* fusion (Fig. 1). Measurement of PHB synthase activities in the particulate crude extracts derived from cells grown in the absence or presence of IPTG confirmed the location of the promoter identified by the S1 nuclease protection assay. Plasmids do95, do186, and do238, which were deleted downstream of the promoter, did not appreciably confer IPTG-independent expression of PHB synthase on the cells (Table 2). In contrast, with plasmid do295 or pSAP33, which harbored the undeleted plasmid, expression of PHB synthase also occurred in the absence of IPTG. As expression was stimulated by IPTG approximately threefold in the latter strains, expression occurred from *phbCp* as well as from *lacZp*.

TABLE 2. Analysis of PHB synthase activity in *E. coli* XL1-Blue harboring hybrid plasmids deleted in the 5' upstream region of *phbC*[a]

Clone	PHB synthase sp act (mU/g of protein) after growth on:	
	LB + IPTG	LB + glucose
Bluescript KS−	<1	<1
pSAP33	380	130
do295	740	220
do95	300	3
do186	650	16
do238	540	15
do181	550	12
do218	37	10

[a] Cells were grown at 37°C in LB medium which was supplemented with 1% (wt/vol) glucose or 1 mM IPTG. Cells were harvested after 48 h of cultivation. PHB synthase activities in the crude cellular extract were determined as described in Materials and Methods. One unit of enzyme activity is defined as the amount required to transform 1 μmol of substrate per min.

Synthesis of truncated PHB synthase protein. It should be emphasized that crude extracts derived from cells of *E. coli* harboring plasmid do181 which were grown in the presence of IPTG expressed active PHB synthase even though 108 nucleotides of the 5' region of *phbC* were deleted. The specific activity of PHB synthase was the same as in clones which were not deleted in the structural gene (e.g., plasmid do238). Even with plasmid do218, in which 300 nucleotides of the 5' region of *phbC* were deleted, PHB synthase was expressed, but the activity was low. Microscopic examination of the cells revealed the presence of inclusion bodies in both clones. As PHB could not be detected in these cells, proteins of the particulate crude extracts were separated in SDS-polyacrylamide gels. As shown in Fig. 3, clones harboring do181 or do218 overexpressed proteins with molecular weights of 56,000 ± 2,000 and 48,000 ± 2,000, respectively. These values correlated very well with the theoretical molecular weights for the fusion gene products encoded by do181 and do218 predicted from the nucleotide sequence, which were 56,492 and 47,588, respectively. The start codon provided by the vector was in frame with the codons of *phbC'* in both plasmids. Both proteins constituted a significant portion (approximately >10%) of total cellular protein.

These results indicated that truncated PHB synthases were synthesized in cells harboring do181 and do218 and that most of the protein was inactive or was deposited in inactive form in the cells. Overexpression was not detected with the other clones (e.g., those harboring do238), and thus, these results indicated that expression of PHB synthase occurred

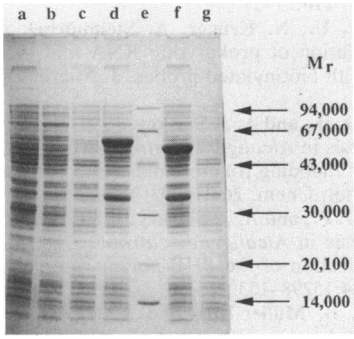

FIG. 3. Overexpression of truncated PHB synthase proteins. Approximately 600 ± 200 μg of crude cellular extracts derived from clones SK2665 (lanes a and b), do181 (lanes c and d), and do218 (lanes f and g) was separated in an SDS-polyacrylamide gel as described in Materials and Methods. Cells were grown in the absence (lanes a, c, and g) or in the presence (lanes b, d, and f) of 1 mM IPTG. The locations of low-molecular-weight calibration kit proteins, which were run in lane e, are indicated by arrows, and the respective molecular weights are given beside the arrows. Proteins were stained with Coomassie brilliant blue.

at a suboptimal level in *E. coli* if the 5′ upstream region of *phbC* was present. One explanation may be a weak ribosome binding sequence, resulting in poor translation initiation. As two translational stop codons (TAGTGA) are located immediately upstream of the *phbC* Shine-Dalgarno sequence, no fusion gene products with vector-encoded information can be synthesized even if a high transcriptional rate resulted in the formation of bulk amounts of polycistronic mRNA. Therefore, translation may be the rate-limiting step in the synthesis of PHB synthase in clones of *E. coli* harboring an unmodified 5′ upstream *phbC* region. In addition to the proteins mentioned above, cells harboring do181 or do218 synthesized a protein exhibiting a molecular weight of approximately 31,000 ± 2,000 (Fig. 3). From the molecular data for the constructs, it might be a fusion protein consisting of the N-terminal regions of 3-ketothiolase and β-ketothiolase.

DISCUSSION

Physiological and molecular studies have revealed the promoter (*phbCp*) and the translation start site of the *A. eutrophus* PHB synthase gene. The 5′ mRNA terminus was mapped 307 bp 5′ upstream from the translational initiation codon. The function of the very long nontranslated 5′ upstream region of the *phbC* mRNA is not known yet. The −35 region (TTGACA) and the −10 region (AACAAT) identified directly upstream of the transcription start site of *phbC* were identical (TTGACA) or very similar (TATAAT) to the corresponding sequences of the *E. coli* σ^{70} consensus promoter sequences (14), and also the spacing between both regions is ideal. It is therefore not surprising that the *A. eutrophus* PHB-biosynthetic genes are expressed in *E. coli* (40, 42) and in most of the pseudomonads investigated recently (43).

To our knowledge, the study of *phbCp* is the first detailed investigation of a promoter constitutively expressed in *A. eutrophus*. The promoter of the *A. eutrophus* alcohol dehydrogenase gene (*adhp*) is only expressed in the wild type if the cells are cultivated under conditions of restricted oxygen supply; in *E. coli*, *adhp* is not expressed (21, 22). Analysis of point mutations of *adhp* revealed that the reason for this nonexpression is most probably the appearance of a G instead of the invariant T in the −10 region of *adhp* (21). A third type of promoter which was identified in *A. eutrophus* resembled the *rpoN*-activatable promoters and was mapped recently upstream of the operon encoding the soluble, NAD-reducing hydrogenase (*hoxp*) (46). It is expected that this type of promoter also controls the expression of genes essential for the catabolism of acetoin (11) and of many other genes in *A. eutrophus* (34).

As the three PHB-biosynthetic genes are most probably organized in one operon in *A. eutrophus*, the promoter which was identified in this study is most likely the promoter not only of *phbC* but of the complete operon. Taking into account (i) the 5′ mRNA terminus, (ii) the length of the three genes and the intergenic regions, and (iii) the location of a potential transcription terminator detected downstream from *phbB* (29), the putative length of the primary transcript of the operon is 4,175 bp. This value correlates very well with the size of the largest hybridization signal (approximately 4.0 kb) which was obtained in Northern (RNA) blots (27).

The overexpression of truncated PHB synthase protein with *lacZp′-′phbC* fusions and the purification of a PHB synthase–β-galactosidase fusion protein by using a *phbC′-′lacZ* fusion indicated useful and practicable strategies provided by recombinant DNA technology to purify quantities of active PHB synthase protein for biochemical studies in the future. Classical biochemical methods which have been used for the purification of this enzyme in the past have not yielded homogeneous and active enzyme preparations (12, 17).

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