JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 774–779 Vol. 178, No. 3
0021-9193/96/$04.00+0

Molecular Analysis of the Poly(3-Hydroxyalkanoate) Synthase Gene from a Methylotrophic Bacterium, *Paracoccus denitrificans*

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Received 31 May 1995/Accepted 14 November 1995

A 3.6-kb *Eco*RI-*Sal*I fragment of *Paracoccus denitrificans* DNA hybridized with a DNA probe carrying the poly(3-hydroxyalkanoate) (PHA) synthase gene (*phaC*) of *Alcaligenes eutrophus*. Nucleotide sequence analysis of this region showed the presence of a 1,872-bp open reading frame (ORF), which corresponded to a polypeptide with a molecular weight of 69,537. Upstream of the ORF, a promoter-like sequence was found. *Escherichia coli* carrying the fusion gene between *lacZ* and the ORF accumulated a level of poly(3-hydroxybutyrate) that was as much as 20 wt% of the cell dry weight in the presence of β-ketothiolase and acetoacetyl-coenzyme A reductase genes of *A. eutrophus*. The ORF was designated $phaC_{Pd}$. A plasmid vector carrying the $phaC_{Pd}$'-'*lacZ* fusion gene downstream of the promoter-like sequence expressed β-galactosidase activity in *P. denitrificans*. When a multicopy and broad-host-range vector carrying the ORF along with the promoter-like sequence was introduced into *P. denitrificans*, the PHA content in the cells increased by twofold compared with cells carrying only a vector sequence.

Poly(3-hydroxyalkanoates) (PHAs) synthesized by bacteria have received great attention as sources for biodegradable plastic materials (4). Poly(3-hydroxybutyrate) [P(3HB)] is the most investigated PHA (4, 8, 16). Interest in a copolyester composed of 3HB and 3-hydroxyvalerate (3HV) [P(3HB-co-3HV)] has been growing recently because its physical properties are suitable for developing biodegradable plastics with wide utility.

Methylotrophic bacteria able to grow on methanol as a sole carbon and energy source are attractive for producing useful metabolites because methanol is an inexpensive feed stock. Some researchers have used methylotrophic bacteria for producing P(3HB) on the basis of biological engineering (30, 31). Recently, we first found that a facultatively methylotrophic bacterium, *Paracoccus denitrificans*, was able to synthesize the P(3HB-co-3HV) copolyester extremely rich in its 3HV unit (more than 98 mol%) in the presence of both methanol and *n*-amyl alcohol (34, 35).

Genes responsible for PHA synthesis have been isolated and characterized from several bacteria (8, 9, 12, 13, 15, 16, 19, 23, 24, 26, 28, 36). In *Alcaligenes eutrophus*, three enzymes, β-ketothiolase, acetoacetyl-coenzyme A (CoA) reductase, and PHA synthase, function to synthesize P(3HB) (15, 18). They are encoded by *phaA*, *phaB*, and *phaC*, respectively. Although these genes are closely located on a chromosomal DNA in *A. eutrophus*, their organization seems to be different in microorganisms. Recently, Valentin et al. isolated and characterized the PHA synthase gene of a methylotrophic bacterium, *Methylobacterium extorquens* (36), which was the first instance in which this was done for methylotrophic bacteria. However, very little is known regarding PHA synthesis at the molecular level. In this study, we report the characterization of the PHA synthase gene of *P. denitrificans* and the increase in synthesis of PHA by its recombinant strain.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used were *P. denitrificans* (ATCC 17741), *Escherichia coli* XL-1 Blue (2) and *E. coli* HB101 (1). Table 1 lists the plasmids used in this study. *P. denitrificans* was aerobically grown in an inorganic salt medium containing 1% (vol/vol) methanol or 0.2% (vol/vol) *n*-amyl alcohol at 30°C (34, 35). *E. coli* was grown at 37°C in Luria-Bertani medium (21). When needed, tetracycline (12.5 μg/ml), kanamycin (25 μg/ml), or ampicillin (50 μg/ml) was added to the medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were used at concentrations of 20 and 40 μg/ml, respectively.

DNA manipulation. Total genomic DNA of *P. denitrificans* was isolated according to the procedure described by Wilson (37). Plasmid DNA isolation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook et al. (21). Conjugative transfer of plasmids into *P. denitrificans* was carried out by a triple mating procedure with *E. coli* HB101 harboring pRK2013 as a helper strain (6). All DNA-manipulating enzymes were used as recommended by the manufacturers.

Southern blotting and colony hybridization analysis. Hybridization was carried out as described by Southern (27). The DNA probe used was a 1.5-kb *Eco*T14I fragment of pSK2665 which contained the *phaC* gene of *A. eutrophus* ($phaC_{Ae}$) (24). Preparation of a digoxigenin-labeled probe and its visualization on the membrane were carried out with the digoxigenin nucleic acid labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Nucleotide sequence analysis. DNA fragments to be sequenced were subcloned into pBluescript II SK$^+$ and used for making serial deletions. DNA sequencing was carried out by the dideoxy chain-termination method described by Sanger et al. (22) with an ALF DNA sequencer (Pharmacia, Uppsala, Sweden). Computer analysis of the nucleotide sequence was performed with SDC-GENETYX genetic information processing software (Software Development Co., Tokyo, Japan).

Electrophoresis of proteins. Proteins were separated by sodium dodecyl sulfate (SDS)-12.5% (wt/vol) polyacrylamide gel electrophoresis as described by Laemmli (11) and were visualized with Coomassie brilliant blue G-250. Protein concentrations were determined by a method described by Lowry et al. (14).

Preparation of partially purified PHA synthase. PHA granules were obtained from a 500-ml culture of *P. denitrificans* as described by Pieper et al. (19) and boiled for 5 min in a mixture of 8% (wt/vol) SDS, 20% (vol/vol) mercaptoethanol, and 40% (vol/vol) glycerol. After centrifugation at 15,000 × *g* for 10 min, the supernatant obtained was used as a partially purified PHA synthase.

Western blot (immunoblot) analysis. The β-galactosidase–PHA synthase fusion protein was recovered from *E. coli* XL-1 Blue harboring pAS1.8 (see below) grown in the presence of 1 mM IPTG. Western blotting was done as described by Burnette (3) with polyvinylidene difluoride membrane. Antibody of the fusion protein was prepared by immunizing a rabbit with the fusion protein as described by Sambrook et al. (21). In immunoblot analysis, the secondary antibody was peroxidase-conjugated anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.). The blot was developed with 4-chloro-1-naphthol (21).

PHA analysis. PHA in the cells was analyzed by gas chromatography (35).

TABLE 1. Plasmids used in this study

Plasmid	Relevant descriptions	Reference or source
pBluescript II SK$^+$ or KS$^+$	Ap^r; *lacPOZ′*; cloning vectors	Stratagene
pRK2013	Km^r Tra$^+$; ColE1 replicon	6
pSK2665	pBluescript II KS$^-$ carrying the *phaC-phaA-phaB* operon of *A. eutrophus*	24
pES3.6	pBluescript II SK$^+$ carrying 3.6-kb *Eco*RI-*Sal*I fragment of *P. denitrificans*	This study
pAS1.8	pBluescript II KS$^+$ derivative; *lacZ′-′phaC$_{Pd}$* fusion	This study
pEE2.0	pBluescript II KS$^+$ derivative; *lacZ′-′phaC$_{Pd}$* fusion	This study
pACYC177	Ap^r Km^r; cloning vector; P15A replicon	20
pAeAB	pACYC177 carrying *phaA-phaB* genes of *A. eutrophus*	This study
pGD926	Tc^r Tra$^+$ Mob$^+$; broad-host-range promoter probing vector	5
pGDA0.9	pGD926 derivative; *phaC$_{Pd}$′-′lacZ* fusion	This study
pBBR1MCS	Cm^r; broad-host-range cloning vector; Tra$^+$ Mob$^+$ IncP	10
pBBRKm	Km^r Cm^s; pBBR1MCS derivative carrying Km^r determinant of Tn*5*	This study
pBBRKm::AS2.7	pBBRKm carrying 2.7-kb *Apa*I-*Sal*I fragment of *P. denitrificans* (Fig. 1)	This study

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number D43764.

RESULTS

Cloning and nucleotide sequence analysis of the *P. denitrificans* PHA synthase gene (*phaC$_{Pd}$*). Southern blot hybridization analysis showed that *Sal*I-*Eco*RI fragments of *P. denitrificans* DNA hybridized with the probe at an approximate size of 3.6 kb. The fragments of approximately that size were recovered from an agarose gel and cloned into pBluescript II SK$^+$. A colony hybridization of *E. coli* transformants with the probe revealed the presence of one positive clone. A recombinant plasmid (pES3.6) harbored by the transformant contained a 3.6-kb insert of *P. denitrificans* DNA. In Fig. 1, the nucleotide sequence of a 2,712-bp region of the 3.6-kb insert is shown. The G+C content of the determined sequence was 66.5 mol%, which was close to that (66 to 68 mol%) reported for *P. denitrificans* DNA (29). From computer analysis for protein-coding regions, one open reading frame (ORF) was found. It started with the ATG start codon at nucleotides 662 to 664 and ended with the TAA stop codon at nucleotides 2,534 to 2,536. The ORF was preceded by a putative translational start signal (5′-GGCGGCG-3′) proposed for several genes from *P. denitrificans* (29) with a reasonable spacing of 8 nucleotides. An inverted repeating structure able to form a potential stem-loop structure was found downstream of the ORF. The free energy of the structure was calculated to be −39.9 kcal (ca. −167 kJ)/mol according to the method described by Tinoco et al. (33).

Gene product encoded by the ORF. The ORF was analyzed for the product encoded. There were two possible start codons of ATG at nucleotide positions 662 and 890. As described below, the ATG codon at the nucleotide position of 662 was selected as the start site of the ORF. The putative gene product translated from the ORF was composed of 624 amino acids and had a molecular weight of 69,537. Figure 2 shows the comparison of a translated gene product of the ORF with known PHA synthases from eight microorganisms (8, 12, 17, 19, 23, 25, 32, 36). Calculated identities ranged from 21 to 56%. The highest identity was obtained with the PHA synthase of *Rhodobacter sphaeroides*; however, the identities with the enzymes of other bacteria were as high as 35%.

Expression of the *lacZ′-′phaC$_{Pd}$* fusion gene in *E. coli* cells. To examine whether the translation product of the ORF could actually exhibit PHA synthase activity, two plasmids carrying *lacZ′-′phaC$_{Pd}$* fusion genes were constructed as follows. One of them, pAS1.8, was constructed by ligating a 1.8-kb *Apa*I-*Sal*I fragment (Fig. 1) of pES3.6 at the *Apa*I and *Sal*I restriction sites of pBluescript II KS$^+$. Another plasmid, pEE2.0, was produced by inserting a filled-in 2.0-kb *Eco*T14I fragment (Fig. 1) of pES3.6 into the *Eco*RV site of pBluescript II KS$^+$. Therefore, pAS1.8 and pEE2.0 should produce β-galactosidase–PHA polymerase fusion proteins with molecular weights of 63,815 and 73,106, respectively. Figure 3A shows the results of SDS-polyacrylamide gel electrophoresis of cell-free lysate from recombinant *E. coli* cells harboring pAS1.8 or pEE2.0. In the presence of IPTG, both plasmids produced extra proteins with molecular weights of 64,000 and 73,000, respectively. These proteins reacted with anti-β-galactosidase–PHA synthase fusion protein antibody (lanes 1 and 3 in Fig. 3B). As shown in Fig. 3B (lane 5), the molecular weight of the PHA synthase produced by *P. denitrificans* was approximately 69,000. This value closely agreed with the molecular weight of the ORF estimated by nucleotide sequence analysis.

The PHA synthase activity of the ORF was examined with *E. coli* cells harboring pAeAB and pAS1.8, which were compatible in a single *E. coli* cell. Since pAeAB contains a *phaA-phaB* operon of *A. eutrophus* downstream from the promoter of the Tc^r gene of pACYC177, it can express β-ketothiolase and acetoacetyl-CoA reductase activities in *E. coli*. Table 2 shows P(3HB) synthesis by recombinant *E. coli* cells grown on sodium lactate, which was used instead of glucose to avoid catabolite repression for the *lacZ* promoter in pAS1.8. P(3HB) accumulated only in the cells harboring pAeAB and pAS1.8.

Analysis of the promoter region of *phaC$_{Pd}$*. In nucleotide sequence analysis of the upstream region of the *phaC$_{Pd}$* gene, the *E. coli* σ^{70}-dependent promoter-like sequences (7) could not be found. Instead, sequences resembling promoter regions of several genes from *P. denitrificans* (29) were found; the most plausible promoter regions for the ORF were 5′-TCGGGC-3′ (nucleotides 554 to 559) for the −35 region and 5′-GCAGGC-3′ (nucleotides 577 to 582) for the −10 region, respectively. These regions were separated by 17 nucleotides. To examine whether these sequences could function as a promoter, a plasmid pGDA0.9 was constructed as follows. A 0.9-kb *Apa*I fragment (nucleotides 1 to 897 in Fig. 1) was inserted into the *Apa*I site of pBluescript II SK$^+$. The recombinant plasmid was digested to recover a 0.9-kb *Sac*I-*Kpn*I fragment. The fragment was treated with T4 DNA polymerase to fill in and was ligated with *Sma*I-digested pBluescript II SK$^+$, which was followed by recovering a 0.9-kb *Bam*HI fragment from the construct. The *Bam*HI fragment was finally inserted into the *Bam*HI site of pGD926 to produce pGDA0.9. If any promoter sequence existed in the upstream region of the *phaC$_{Pd}$* gene, pGDA0.9

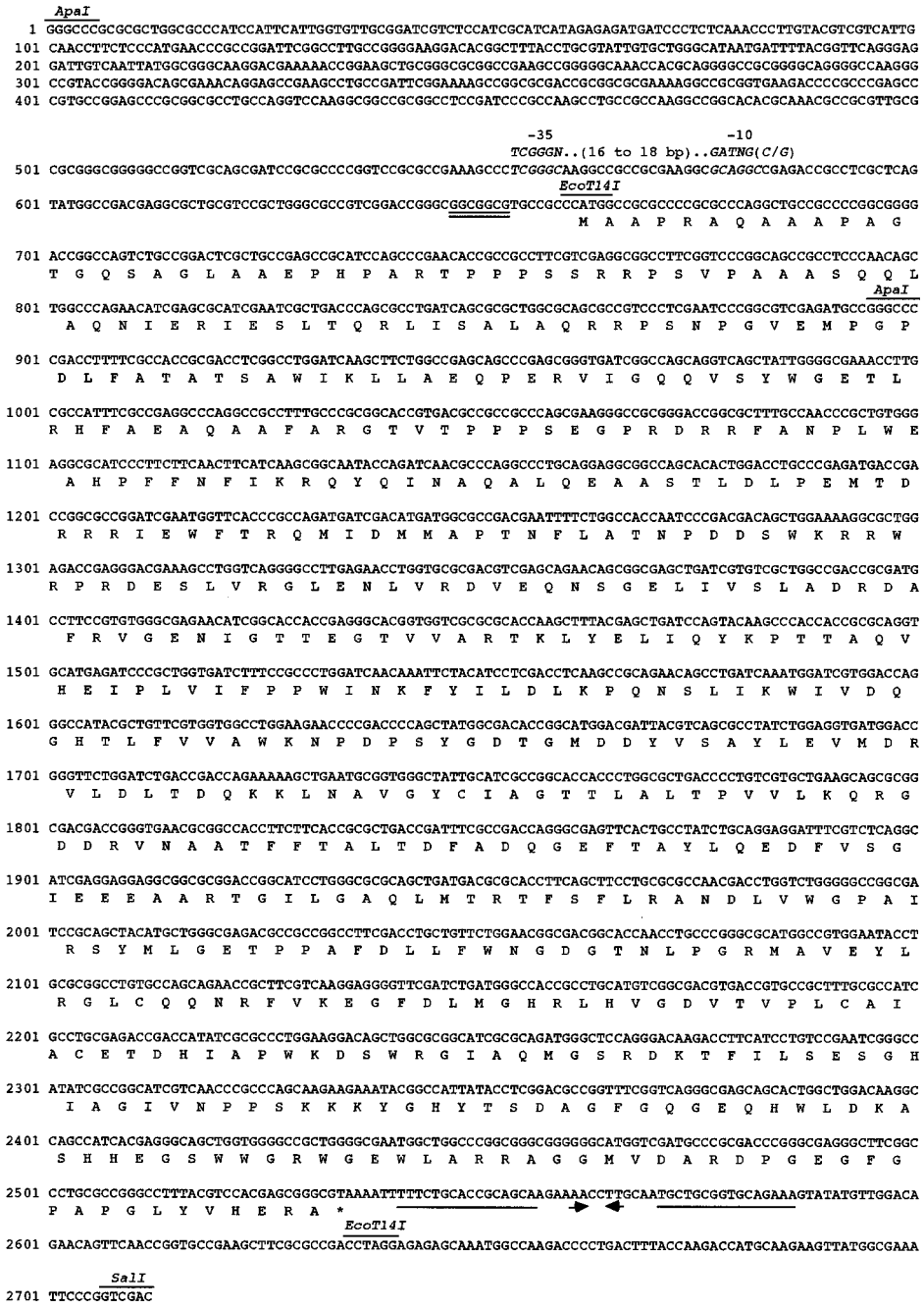

FIG. 1. Nucleotide sequence of a 2,712-bp region containing the *phaC*$_{Pd}$ gene, along with the deduced amino acid sequence. The potential ribosomal binding site is doubly underlined. The facing arrows indicate a potential stem-loop structure. Possible −35 and −10 regions are in italics, along with consensus promoter sequences of *P. denitrificans.*

should have produced a fused β-galactosidase as a reporter enzyme in *P. denitrificans*. When the plasmid was introduced into *P. denitrificans*, transconjugants exhibited blue on an agar plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (data not shown).

PHA production from *n*-amyl alcohol by recombinant *P. denitrificans.* To examine the effect of an increased amount of the *phaC*$_{Pd}$ gene on the PHA synthesis in *P. denitrificans*, pBBRKm::AS2.7 was constructed by introducing the 2.7-kb *Apa*I-*Sal*I fragment of pES3.6 into a multicopy and broad-host-range plasmid vector, pBBRKm, at its multiple cloning sites. Table 3 shows PHA production from *n*-amyl alcohol by the *P. denitrificans* recombinant cells. Since the PHA samples to be analyzed were obtained from exponentially growing cells, PHA was synthesized in a growth-associated fashion. The PHA content in the cells harboring pBBRKm::AS2.7 was 1.9-fold higher than that in the cells harboring pBBRKm. The compositions of the synthesized PHAs were almost the same for all strains examined; the PHAs were extremely rich in the 3HV unit.

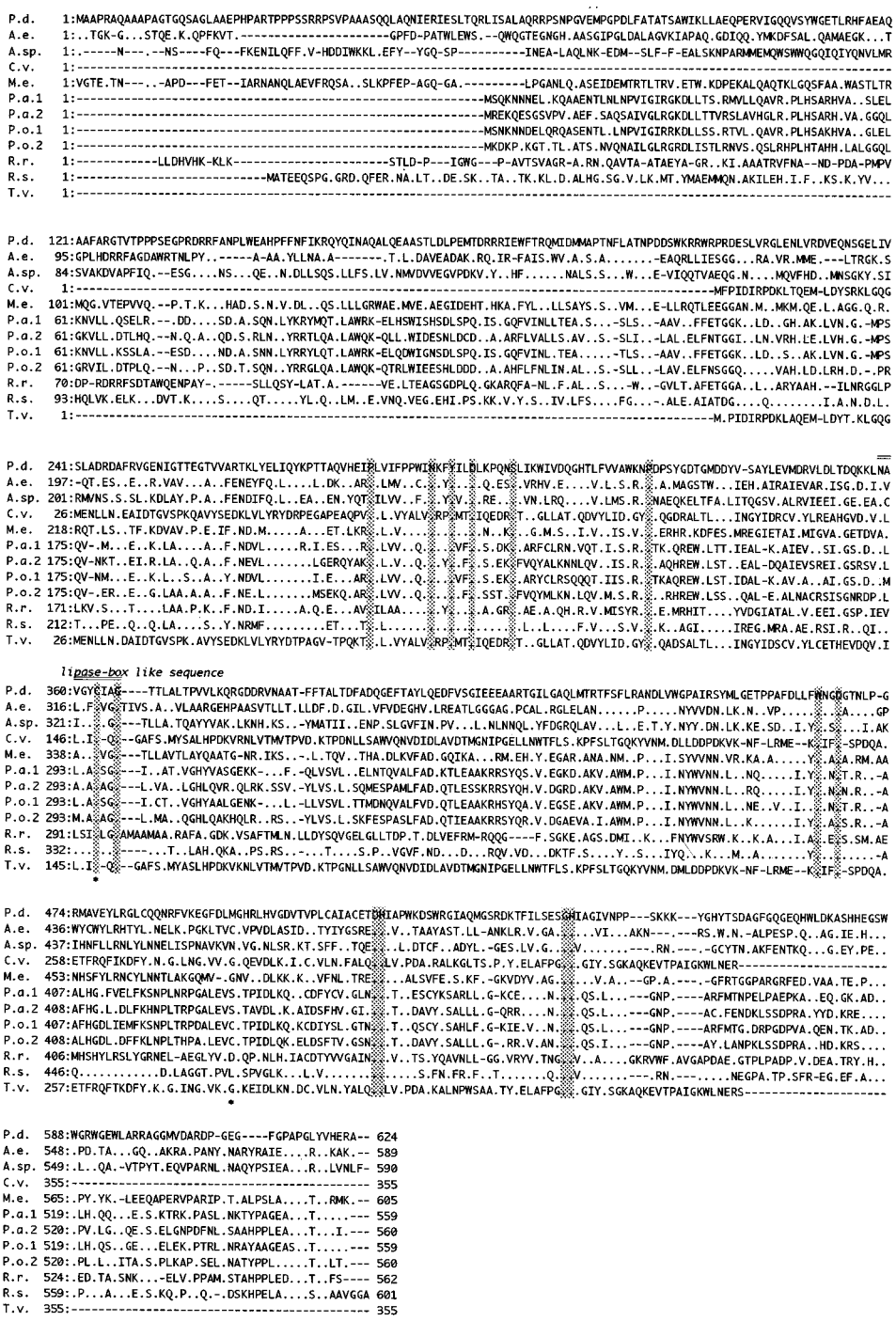

FIG. 2. Alignment of the deduced amino acid sequence of the PHA synthase from *P. denitrificans* (P.d.) with those from *A. eutrophus* (A.e.) (16, 24), *Acinetobacter* sp. (A.sp.) (23), *Chromatium vinosum* (C.v.) (12), *M. extorquens* (M.e.) (36), *Pseudomonas aeruginosa* (P.a. 1 and 2) (32), *P. oleovorans* (P.o. 1 and 2) (8), *Rhodococcus ruber* (R.r.) (19), *R. sphaeroides* (R.s.) (9), and *Thiocystis violacea* (T.v.) (13). Dots, amino acids identical to the *P. denitrificans* sequence; dashes, gaps introduced to maximize alignment of the sequences; shading, identical residues present in all the sequences. Cysteine residues marked by asterisks and the methionine residue at position 77 in the *P. denitrificans* sequence are discussed in the text. A lipase box-like sequence is indicated by a double line above the sequence.

DISCUSSION

In the nucleotide sequence analysis of the *P. denitrificans* PHA synthase gene, two possible initiation codons were found. Western blot analysis showed that the ATG codon at nucleotide 662 was more plausible as the initiation codon of $phaC_{Pd}$ gene. Since both pAS1.8 and pEE2.0 could produce P(3HB) in *E. coli* cells, it is certain that the region needed for expressing the activity of the enzyme is located after the 77th amino acid residue of the protein. When P(3HB) synthesis in *E. coli* cells was examined with pAS1.8, approximately 20 wt% of P(3HB) was accumulated in the cells. However, when pEE2.0 was used, the content of PHB accumulated was only 6 wt% (data not

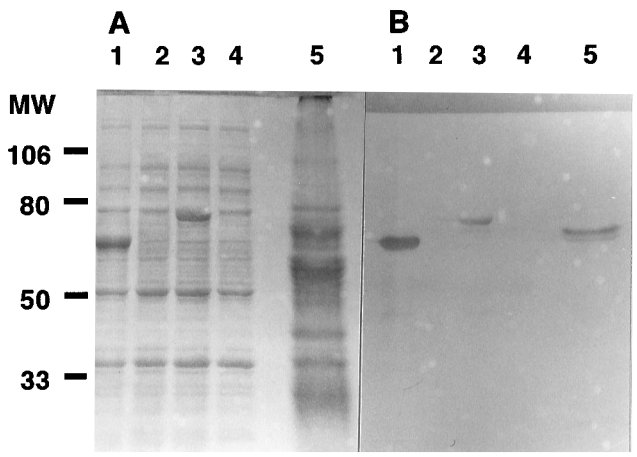

FIG. 3. Expression of a β-galactosidase–PHA synthase fusion protein in *E. coli* (A) and Western blot analysis with anti-β-galactosidase–PHA synthase fusion protein antibody (B). In lanes 1 and 3, *E. coli* cells were grown in the presence of IPTG, whereas in lanes 2 and 4, IPTG was omitted. As a plasmid, pAS1.8 (lanes 1 and 2) or pEE2.0 (lanes 3 and 4) was used. Proteins dissociated from PHA granules of *P. denitrificans* were loaded in lane 5. Molecular weights (MW) are indicated to the left (in thousands).

shown). Although it is difficult to clearly explain this phenomenon, the N-terminal region of *P. denitrificans* PHA synthase might influence the expression of the enzyme activity.

The P(3HB) synthesis from 3-hydroxybutyryl-CoA includes the formation of an acyl-S enzyme intermediate and the transesterification to a primer acceptor (8). Two cysteine residues conserved in the *A. eutrophus* and *Pseudomonas oleovorans* PHA synthases (marked with asterisks in Fig. 2) have been proposed to be involved in the two catalytic steps (8). The cysteine residue at the amino acid position of 363 was also conserved in the *P. denitrificans* PHA synthase, whereas another cysteine residue was absent at the corresponding amino acid position of 497. Therefore, the latter residue might not be included in PHA synthesis. The conserved Cys-363 was found in the lipase box-like sequence (Fig. 2). It is interesting that the lipase box-like sequence of the *R. sphaeroides* PHA synthase was completely identical to that of the *P. denitrificans* enzyme because the two enzymes were very homologous.

The existence of the promoter for the $phaC_{Pd}$ gene was demonstrated upstream of the gene. Sequences coding for β-ketothiolase and acetoacetyl reductase could not be found upstream and downstream of the $phaC_{Pd}$ gene. Very recently, we found that they were organized very closely but were located separately from the $phaC_{Pd}$ gene on the chromosomal DNA of *P. denitrificans*. Therefore, the $phaC_{Pd}$ gene must be expressed independently in this bacterium. *P. denitrificans* produced a copolyester of P(3HB-co-3HV) extremely rich in the 3HV unit in the presence of methanol plus *n*-amyl alcohol (35). Here, we examined the effect of an increased level of the $phaC_{Pd}$ gene on PHA synthesis from *n*-amyl alcohol. As expected, the PHA content in a recombinant *P. denitrificans* strain harboring pBBRKm::AS2.7 increased by approximately twofold. This result suggests that more effective PHA synthesis with a recombinant *P. denitrificans* strain should become possible by employing an elaborate cultivation technique.

TABLE 2. P(3HB) synthetic activity in recombinant *E. coli* XL-1 Blue

Plasmid	Relevant marker(s)	Accumulation of P(3HB) (% wt)[a]
pAeAB + pAS1.8	$phaAB_{Ae}$ and $lacZ'$-'$phaC_{Pd}$	18.9
pAeAB + pBluescript II KS$^+$	$phaAB_{Ae}$	<0.1
pACYC177 + pAS1.8	$lacZ'$-'$phaC_{Pd}$	<0.1
pACYC177 + pBluescript II KS$^+$		<0.1

[a] P(3HB) content was analyzed for the cells grown on Luria-Bertani medium containing 2% (wt/vol) sodium lactate and 1 mM IPTG for 50 h.

TABLE 3. PHA production from *n*-amyl alcohol by recombinant *P. denitrificans*

Plasmid	Cultivation time (h)[a]	Dry cell concn (g/liter)	PHA content (wt%)	3HV content in the polyester (wt%)
None	22.8	0.84	24.3	95.9
pBBRKm	23.5	0.88	26.1	96.2
pBBRKm::AS2.7	28.8	0.77	48.4	94.0

[a] Cells were harvested at the late exponential growth phase.

ACKNOWLEDGMENTS

We thank A. Steinbüchel, D. R. Helinski, and M. E. Kovach for kindly providing *E. coli* strains harboring pSK2665, pGD926, and pBBR1MCS, respectively.

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