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Identification and Characterization of Two *Alcaligenes eutrophus* Gene Loci Relevant to the Poly(β-Hydroxybutyric Acid)-Leaky Phenotype Which Exhibit Homology to *ptsH* and *ptsI* of *Escherichia coli*

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From genomic libraries of *Alcaligenes eutrophus* H16 in λL47 and in pVK100, we cloned DNA fragments which restored the wild-type phenotype to poly(β-hydroxybutyric acid) (PHB)-leaky mutants derived from strains H16 and JMP222. The nucleotide sequence analysis of a 4.5-kb region of one of these fragments revealed two adjacent open reading frames (ORF) which are relevant for the expression of the PHB-leaky phenotype. The 1,799-bp ORF1 represented a gene which was referred to as *phbI*. The amino acid sequence of the putative protein I (M_r, 65,167), which was deduced from *phbI*, exhibited 38.9% identity with the primary structure of enzyme I of the *Escherichia coli* phosphoenolpyruvate:carbohydrate phosphotransferase system (PEP-PTS). The upstream 579-bp ORF2 was separated by 50 bp from ORF1. It included the 270-bp *phbH* gene which encoded protein H (M_r, 9,469). This protein exhibited 34.9% identity to the HPr protein of the *E. coli* PEP-PTS. Insertions of Tn*5* in different PHB-leaky mutants were mapped at eight different positions in *phbI* and at one position in *phbH*. Mutants defective in *phbH* or *phbI* exhibited no pleiotropic effects and were not altered with respect to the utilization of fructose. However, PHB was degraded at a higher rate in the stationary growth phase. The functions of these HPr- and enzyme I-like proteins in the metabolism of PHB are still unknown. Evidence for the involvement of these proteins in regulation of the metabolism of intracellular PHB was obtained, and a hypothetical model is proposed.

The ability to accumulate poly(β-hydroxybutyric acid) (PHB), functioning as a carbon and/or energy source or as a sink for reducing equivalents, is widespread among prokaryotic organisms. The hydrogen-oxidizing bacterium *Alcaligenes eutrophus* accumulates PHB to more than 80% (wt/wt) of the cellular dry weight (48). PHB and related polyesters are already produced industrially on a small scale by *A. eutrophus* and are distributed under the trade name Biopol (7, 30). Recently, two classes of transposon-induced mutants of *A. eutrophus*, which are affected in the accumulation of PHB, were isolated (55). PHB-negative mutants were completely impaired in the synthesis of PHB; in this class of mutants Tn*5*::*mob* (56) mapped within the structural gene of PHB synthase. This fragment encodes the complete *A. eutrophus* PHB-synthetic pathway and harbors the genes for the biosynthetic β-ketothiolase (*phbA*), NADPH-dependent acetoacetyl coenzyme A reductase (*phbB*), and PHB-synthase (*phbC*) (39, 40, 55, 58). The genes are organized in one operon (*phbCAB*) which is transcribed from a σ^{70}-dependent promoter (54, 60). DNA fragments harboring this operon conferred the ability to accumulate PHB to *Escherichia coli* and to many pseudomonads belonging to rRNA homology group I (55, 58, 61, 63, 64).

Transposon-induced mutants, belonging to the second class, accumulated less PHB than the wild type. The phenotype of this mutant class was referred to as PHB-leaky. PHB-leaky mutants exhibited activities of all three PHB-biosynthetic enzymes; there was also no evidence that isoenzymes of the biosynthetic β-ketothiolase (25) or of the acetoacetyl coenzyme A reductase (26) were affected in these mutants. Measurements with reconstituted enzyme systems had shown that both ketothiolases can contribute to PHB synthesis in *A. eutrophus* (26). Theoretical considerations based on the experimental data indicated that the NADPH-dependent acetoacetyl coenzyme A reductase is not essential for the synthesis of PHB in this bacterium (60). Tn*5*::*mob* has not inserted into the PHB synthase operon or in adjacent regions, and the phenotype of the wild type was not restored upon the transfer of this operon (55). Therefore, it was concluded that gene loci different from that encoding the complete PHB-biosynthetic pathway are needed for maximum accumulation of PHB in *A. eutrophus*. However, the function of these gene loci remained unclear. In this study we cloned a single DNA fragment which restored the phenotype of the wild type in PHB-leaky mutants and identified two gene loci which are insertionally inactivated in PHB-leaky mutants.

(Part of this work was presented at the NATO Advanced Research Workshop, New Biosynthetic Biodegradable Polymers of Industrial Interest from Microorganisms, Sitges, Spain, 28 May 1990 (62); at the International Symposium on Biodegradable Polymers, Tokyo, Japan, 30 October 1990, and at the 5th Leipziger Biotechnologie Symposium, Leipzig, Germany, 12 December 1990.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *A. eutrophus* and *E. coli*, as well as the plasmids and bacteriophage used in this study, are listed in Tables 1 and 2.

Growth of bacteria and analysis of PHB content. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium (45). *A. eutrophus* was grown at 30°C either in a complex medium of nutrient broth (NB) (0.8%, wt/vol) or in a mineral salts

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TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or bacteriophage	Relevant characteristics	Source or reference
A. eutrophus		
H16	Wild type, prototrophic	DSM 428, ATCC 17699
H16-PHB$^-$4	PHB-negative mutant of H16	50; DSM 541
PSI	Tn*5*-induced PHB-negative mutant of a Sm^r strain of H16	55
JMP222	Wild type, prototrophic	15
JMP222-PHB$^-$180	PHB-negative mutant of JMP222	A. Timm, Göttingen
JMP222-PHB$^-$151	PHB-leaky mutant of JMP222	A. Timm, Göttingen
E. coli		
DH1	*recA1*, auxotrophic for proline	23
S17-1	*recA* and *tra* genes of plasmid RP4 are integrated into the chromosome, auxotrophic for proline and thiamine	57
WL87	*recBC*	Amersham Buchler
WL95	*metB supE supF hsdR trpR* P2	Amersham Buchler
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q} M15 Tn*10*(Tet)]	5
L191	F$^-$ *argG6 galT6 gatR49 gntApo49 hisG1 lacY1 malT1 metB1 ptsI191 mtlApo50 rpsL104 supE44 thi-1 tonA2*	35
Plasmids		
pHC79	Cosmid, Tc^r Ap^r	29
pBR325	Ap^r Cm^r Tc^r	3
pMC1403	Ap^r *lacZ′Y*	8
pUC9-1	Ap^r *lacPOZ′*	24
pUCCM9-11	Ap^r Cm^r	This study
pVK100	Cosmid, Tc^r Km^r	33
pVK101	Tc^r Km^r	33
pVK101::PP1	*phbCAB*	55
pHP1014	Tc^r Km^r Cm^r	This study
pHP1016	Tc^r Km^r Cm^r	17
pBluescript SK$^-$	Ap^r *lacPOZ′*, T7 and T3 promoter	Stratagene
pBluescript KS$^-$	Ap^r *lacPOZ′*, T7 and T3 promoter	Stratagene
pHZ1	pMC1403 derivative, *phbH′-′lacZ*	This study
pHZ2	pVK101 derivative, *phbH′-′lacZ*	This study
pBH4550	pVK101::BH4550, *phbHI*	This study
pBL7502	pVK101::BL7502, *phbI*	This study
Bacteriophage		
λL47		36

medium (MM) (49). To allow extensive accumulation of PHB, the concentration of NH_4Cl in the MM was reduced to 0.05 or 0.005% (wt/vol). The PHB content in lyophilized cells was determined by gas chromatography by the method of Brandl et al. (4) as described in detail recently (64).

Determination of metabolites. Pyruvate, gluconate, and D-(−)β-hydroxybutyrate were determined photometrically in cell-free fermentation broths by the methods of Czok and Lamprecht (10), Möllering and Bergmeyer (37), and Williamson and Mellanby (66), respectively, following the reduction or oxidation of pyridine nucleotides.

TABLE 2. Classes of PHB-leaky mutants[a]

Mutant class	Mutant strain(s)	Fragment which restored the wild-type phenotype
A	H1070, H1071, H1472, H1476, H1477, H1480, H1481, H1483, H1484, H1488, H1489, H2261, H2265, H2270, H2274, H2278	BL7502
B	H1486	PE1
C	H1474, H1482, H1485, H2262, H2271, H2272, H2273, H2275, H1479	Unknown (neither BL7502, PE1, nor PP1)

[a] Some of the PHB-leaky mutants are mentioned in Table 1 of reference 55. To standardize designation of these mutants, we have renamed them as follows: H1470 (formerly RT05), H1477 (RT16), H1483 (RT06), H1486 (RT01), H2262 (RT18), and H2275 (RT49).

Isolation and analysis of DNA. Total genomic DNA, plasmids, and λ DNA were isolated by standard procedures (45). DNA restriction fragments were isolated from agarose gels by electroelution into a sodium acetate solution in an apparatus obtained from Biometra, Göttingen, Germany. Isolated DNA was digested with various restriction endonucleases under the conditions described by the manufacturer. DNA restriction fragments were separated in gels containing 0.8 to 2.0% (wt/vol) agarose in TBE buffer (45).

Hybridization experiments. Denatured DNA was transferred from agarose gels to BA85 nitrocellulose filters (pore size, 0.45 μm; Schleicher and Schüll, Dassel, Germany) by vacuum blotting in the VacuGene blotting apparatus (Pharmacia-LKB, Uppsala, Sweden) under the conditions described by the manufacturer. Conditions for the hybridization of DNA with bio-11-dUTP-labeled probes and for the detection of biotinylated DNA have been described in detail previously (34). λL47 libraries were screened on filters as described by the manufacturer of the DNA detection kit (Bethesda Research Laboratories).

Transfer of DNA. Transformation of *E. coli* was done by the $CaCl_2$ procedure as described by Hanahan (23). Matings of *A. eutrophus* (recipient) with *E. coli* S17-1 (donor) harboring hybrid donor plasmids were performed on solidified NB medium as described by Friedrich et al. (16).

Construction of cosmid and lambda L47 libraries. Genomic DNA was partially digested with restriction endonucleases, ligated to linearized vector DNA, and packaged with lambda coat proteins by using an in vitro packaging kit. Phage particle were transfected into *E. coli* DH1 as described by Hohn and Collins (29).

Manipulation of DNA molecules. Recessed 3′ ends of restricted DNA molecules were filled in with the Klenow enzyme by following information provided by the manufacturer. Protruding single-stranded ends of restricted DNA were removed by incubating approximately 1 μg of DNA for 30 min at 37°C in a total volume of 10 μl of 50 mM sodium acetate (pH 4.7), 300 mM NaCl, 10 mM zinc acetate, and 8 U of S1 nuclease.

Construction of pHP1014 and pHP1016. Derivatives of the plasmid pVK101 were constructed, which harbor the chloramphenicol acetyltransferase (*cat*) gene from pBR325 with one single *Eco*RI site. The 2.4-kb *Pst*I-*Hin*dIII fragment of pBR325 was treated with *Bal* 31 and was ligated to dephosphorylated pUC9-1 DNA which had been treated with *Pst*I and then with S1 nuclease. Ligated DNA was transformed to *E. coli* JM83, and chloramphenicol-plus-ampicillin-resistant clones were analyzed for the presence of a small *Hin*dIII-*Sal*I-fragment. One of the clones harbored a 4.3-kb hybrid plasmid, which was designated pUCCM9-11. From pUCCM9-11 a 1.6-kbp *Hin*dIII-*Sal*I fragment was isolated, and recessed ends were filled in with Klenow polymerase; derivative fragments were ligated to pVK101 DNA which had been linearized with *Eco*RI and treated with S1 nuclease, and chloramphenicol-plus-tetracycline-resistant clones of *E. coli* were selected. Two recombinant plasmids, which were referred to as pHP1014 and pHP1016 and which harbored the 1.6-kb chloramphenicol resistance gene block in opposite directions, were isolated. The new derivatives of pVK101 provided a unique cleavage site for *Eco*RI within the *cat* gene, which can be inactivated by a recombinational event.

DNA sequence analysis and analysis of sequence data. DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (46) with alkali-denatured double-stranded plasmid DNA, 7-deazaguanosine 5′-triphosphate instead of dGTP, and [α-^{35}S]dATP by using a T7 polymerase kit as specified by the manufacturer. Universal and synthetic oligonucleotides were used as primers. Nucleic acid sequence data and deduced amino acid sequences were analyzed with the Sequence Analysis Software Package (version 6.2, June 1990) according to Devereux et al. (14).

Synthesis of oligonucleotides. Synthetic oligonucleotides were synthesized in 0.2-mmol portions from deoxynucleoside phosphoramidites in a Gene Assembler Plus apparatus as specified by the manufacturer (Pharmacia-LKB). Oligonucleotides were released from the support matrix, and protection groups were removed by a 15-h incubation at 55°C in 25% (vol/vol) ammonium. Oligonucleotides were finally purified by passage through a NAP-10 column (Pharmacia-LKB).

Isolation of *lacZ* fusion proteins and N-terminal sequence analysis. The fusion protein was purified from crude ultracentrifugal supernatants by chromatography on *p*-aminophenyl-β-D-thiogalactopyranoside (APTG)–Sepharose columns (65) obtained from Mobitec, Göttingen, Germany. The sequence analysis was performed with a 477A pulsed liquid phase protein peptide sequencer (28) and a 120A on-line phenolthiohydantoin amino acid analyzer (44) as specified by the manufacturer (Applied Biosystems, Weiterstadt, Germany).

Determination of the 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 Sambrook et al. (45), and the S1 nuclease reactions were conducted by the method described by Aldea et al. (1). Total RNA was isolated as described by Oelmüller et al. (38). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with pBH4550 DNA as a template. For the annealing reactions, two different oligonucleotides (5′-GCGCCGTTGTCCTC GCA-3′ and 5′-TTGATGATGGTGGTGT-3′), which were complementary to the regions from positions 796 to 812 and from 1065 to 1080 (see Fig. 2), were used for ^{35}S labeling.

Chemicals. Restriction endonucleases, biotin-11-dUTP, the nick translation kit, the DNA detection kit, T4 DNA ligase, lambda DNA, and DNA-modifying enzymes were obtained from C. F. Boehringer & Soehne, Mannheim, Germany, or from GIBCO/BRL-Bethesda Research Laboratories GmbH, Eggenstein, Germany. Agarose type NA and RNase-free DNase were purchased from Pharmacia.

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported here have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession no. M69036.

RESULTS

Phenotypic characterization of PHB-leaky mutants. Mutants of *A. eutrophus* exhibiting the phenotype PHB-leaky grew normally on fructose or on any other substrate tested. On mineral agar plates, which allowed accumulation of PHB from fructose or gluconate, the opacity of the colonies was intermediate between the opacity exhibited by the wild type and that of PHB-negative mutants which were completely impaired in the synthesis of PHB. After prolonged incubation, the opacity became even less, indicating that the amount of the material causing the opacity was diminishing.

Analysis of cells in liquid medium clearly showed that PHB was accumulated only at intermediate levels at the beginning of the storage phase. After the carbon source of the medium had been exhausted, the PHB content of the cells began to drop much more rapidly in PHB-leaky mutants than in the wild type (Fig. 1). The decrease in the PHB content was generally much greater in cells of *A. eutrophus* JMP222 or of a PHB-leaky mutant derived from this strain (e.g., strain PHB$^-$151) than in cells of *A. eutrophus* H16 or of derivatives (e.g., strain RT4). These results indicated that the mobilization rather than the synthesis of PHB was affected in PHB-leaky mutants and were consistent with the finding that PHB-leaky mutants synthesized the enzymes for the PHB-biosynthetic pathway at almost normal levels (55). In contrast to PHB-negative mutants, which excreted large amounts of pyruvate into the medium if the cells were cultivated under conditions allowing accumulation of PHB in the wild type (59), pyruvate was detected only at much lower concentrations in the medium of PHB-leaky mutants of H16 (maximum level, 2 mM) or of JMP222 (approximately 7.5 mM [Fig. 1]). 3-Hydroxybutyrate was not detected with PHB$^-$151. A physiological rationale for the different behavior of the wild type and of the PHB-leaky mutants with

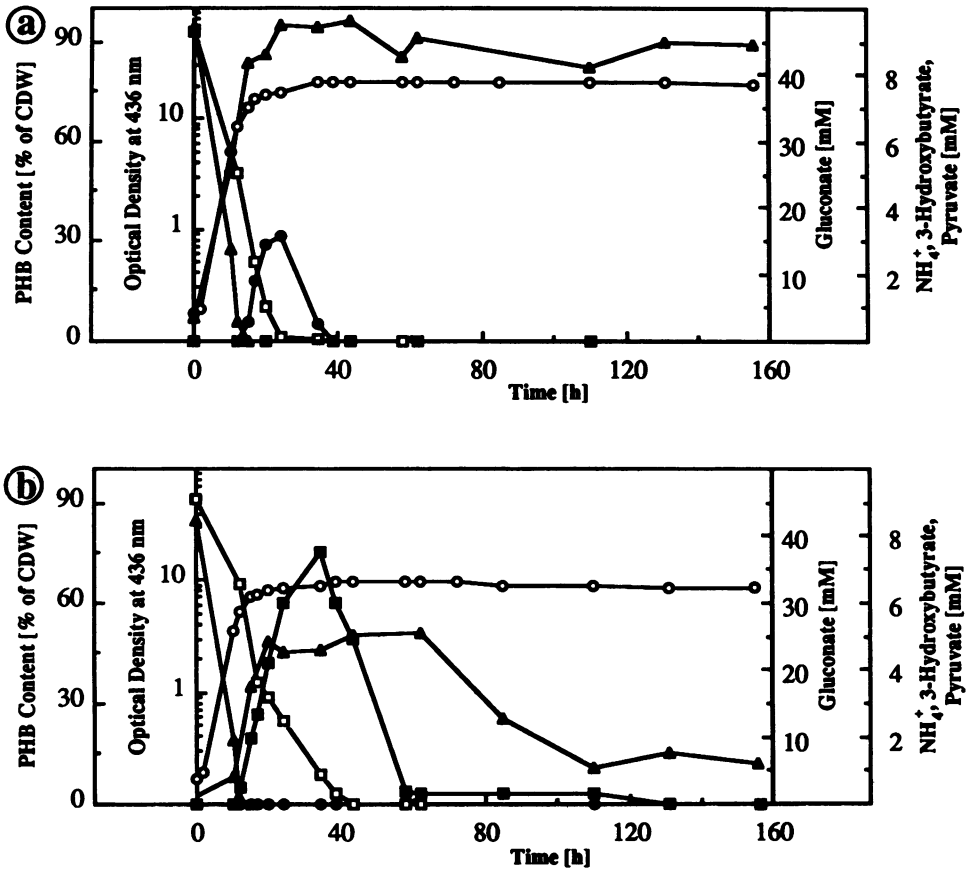

FIG. 1. Synthesis and mobilization of PHB in *A. eutrophus* during fermentation on gluconate. MM (8 liters) containing 0.05% (wt/vol) ammonium chloride and 1.0% (wt/vol) sodium gluconate were inoculated with a 300-ml suspension of cells of *A. eutrophus* JMP222 (a) or *A. eutrophus* JMP222-PHB$^-$151 (b) which had been grown in NB medium for 24 h. Cells were aerobically (1200 ml of air/liter of medium/min) cultivated for 155 h, and growth was monitored in a Zeiss photometer. Samples were withdrawn as indicated; cells were analyzed for PHB content, and cell-free supernatants were analyzed for ammonium, gluconate, pyruvate, and 3-hydroxybutyrate as described in Materials and Methods. Symbols: ○, optical density; △, PHB; □, gluconate; ▲, ammonium; ■, pyruvate; ●, 3-hydroxybutyrate.

respect to the excretion of these metabolites cannot be provided.

Identification of genomic fragments relevant for the phenotype PHB-leaky. To determine whether the insertions of Tn*5*::*mob* (56) were restricted to one single region in the genomes of PHB-leaky mutants of *A. eutrophus*, we cloned Tn*5*-labeled *Eco*RI fragments from 19 PHB-leaky mutants in the cosmid pHC79. The ligation mixtures were packaged with lambda coat proteins and transfected into *E. coli* DH1. Recombinant clones carrying fragments with Tn*5*::*mob* were selected by plating on LB medium which contained ampicillin plus kanamycin. The hybrid cosmids of eight recombinant clones obtained from each packaging reaction were isolated and digested with *Eco*RI. All recombinant cosmids harbored a 6.4-kb fragment (pHC79 DNA), one or more *Eco*RI fragments of various sizes, and one fragment of approximately 8.4 or 9.5 kb. The latter harbored Tn*5*::*mob*, as was shown by hybridization with a Tn*5*::*mob*-specific DNA probe. This indicated that the sizes of the native *Eco*RI fragments, which are insertionally inactivated in PHB-leaky mutants, were approximately 2.0 or 0.9 kb, considering that the size of Tn*5*::*mob* is 7.5 kb (56) and that the transposon has no restriction site for *Eco*RI (32).

Cloning of native genomic fragments. The Tn*5*::*mob*-harboring *Eco*RI restriction fragment from mutant strain H1489, which was referred to as RT4, was biotinylated and used to detect the native 2.0-kb *Eco*RI fragment in a λL47 gene bank of *A. eutrophus* H16 in *E. coli* WL87, which was derived from genomic DNA incompletely digested with *Eco*RI. One positive recombinant phage was purified to homogeneity, and a 2.0-kb *Eco*RI restriction fragment, which was referred to as EI2045, was isolated. It was subsequently ligated to linearized pHP1016 DNA and transferred to *E. coli* S17-1.

Hybrid plasmids of pHP1016, which harbored RI2045, did not complement PHB-leaky mutants in *trans*. As RI2045 was rather small, it probably did not harbor all of the essential genetic information. Hybridization experiments with biotinylated RI2045 DNA revealed that RI2045 was part of a 7.5-kb *Bgl*II restriction fragment which was referred to as BL7502 (Fig. 2). This fragment, which contains approximately 960 bp of λ DNA, was isolated and ligated to the *Bgl*II site of pVK101. The resulting plasmid was referred to as pBL7502. As shown in Table 2, plasmid pBL7502 restored the phenotype of the wild type in *trans* in many Tn*5*-induced PHB-leaky mutants derived from strain H16. In addition, strain PHB$^-$151, a PHB-leaky mutant derived from strain JMP222, was complemented.

Because the nucleotide sequence analysis (see below) of part of BL7502 indicated that the region relevant for the PHB-leaky gene locus was not complete, a gene bank of

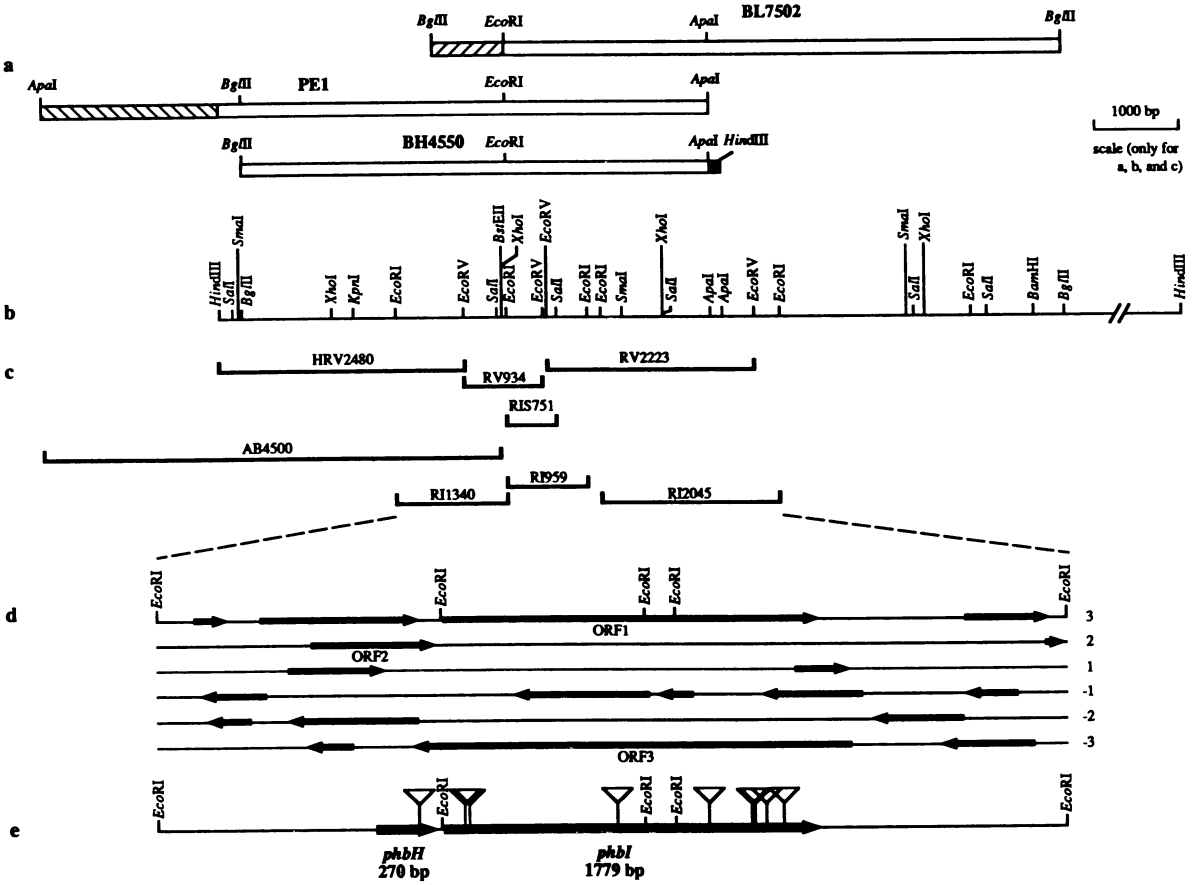

FIG. 2. Restriction endonuclease sites and DNA sequencing strategy. (a) DNA fragments complementing PHB-leaky mutants: ▨, λL47 DNA; ▧, pVK100 DNA; ■, pBluescript SK⁻ DNA. (b) Physical map as determined by restriction analysis. (c) Subfragments relevant for nucleotide sequence analysis. (d) Positions and orientations of ORFs with more than 150 nucleotides indentified in this study (numbers indicate the six possible frames). (e) Putative orientation of *phbH* and *phbI* and locations of transposon insertions as determined by sequence analysis of mutant DNA fragments.

*Hin*dIII-digested genomic DNA of strain H16 in the broad-host-range cosmid pVK100 was prepared in *E. coli* S17-1. Tetracycline-resistant transductants were transferred with toothpicks from LB-tetracycline agar plates to a lawn of PHB⁻151 cells on mineral agar plates containing 0.05% (wt/vol) NH_4Cl, 0.5% (wt/vol) sodium gluconate, and 12.5 μg of tetracycline per ml, thus allowing accumulation of PHB in the cells. Transconjugants, which formed whitish opaque colonies, appeared at a frequency of approximately 1 per 200. The hybrid cosmid isolated from one of them harbored a *Hin*dIII restriction fragment of approximately 20 kb. This plasmid complemented not only PHB⁻151 but also all of the PHB-leaky mutants of H16 which were complemented by pBL7502. In addition, the wild-type phenotype was restored in the PHB-leaky mutant H1486. This mutant was not complemented by pBL7502. From the hybrid plasmid harboring fragment PE1 and Bluescript SK⁻ DNA, a 4.55-kb *Hin*dIII-*Bgl*II subfragment (BH4550 [Fig. 2]) was cloned into pVK101 DNA which had been treated with *Hin*dIII plus *Bgl*II. The resulting plasmid, which was referred to as pBH4550, restored the wild-type phenotype in PHB-leaky mutants. Either fragment cloned in this study restored the ability to synthesize PHB in PHB-negative mutants such as strains PHB⁻4, PSI (derivatives of H16), or PHB⁻180 (derivative of JMP222).

Determination of the nucleotide sequence. Fragment RI2045 was cloned in both orientations into the Bluescript vector KS⁻. To obtain unidirectional nested deletions, both resulting hybrid plasmids were digested with *Hin*dIII and *Kpn*I. Subsequently, the DNA was treated with exonuclease III, and overhanging single-stranded DNA was removed with mung bean nuclease (27). Deleted plasmids were isolated from recombinant strains of *E. coli* XL1-Blue. The nucleotide sequence of RI2045 was obtained from overlapping partial sequences determined for both strands by using the dideoxy-chain termination method and universal primers. The nucleotide sequence obtained for RI2045 was extended in the 5′ direction to approximately 4.5 kb by subjecting Bluescript vectors harboring the 7.5-kb *Apa*I restriction fragment PE1 (Fig. 2), which was derived from the hybrid cosmid harboring the 20-kb *Hin*dIII fragment as well as subfragments HRV2480, RV934, RV2223, RIS751, RI1340, and RI959 (Fig. 2), to sequencing with synthetic oligonucleotides as primers (Fig. 2 and 3).

Several open reading frames (ORFs) were identified (Fig. 2). ORF1 (1,779 bp) and ORF2 (579 bp), which were separated by 50 nucleotides, were of special interest with respect to PHB metabolism because they covered all identified insertions of Tn*5* in PHB-leaky mutants. ORF3 (2,073 bp) was the most likely alternative candidate, as it also included

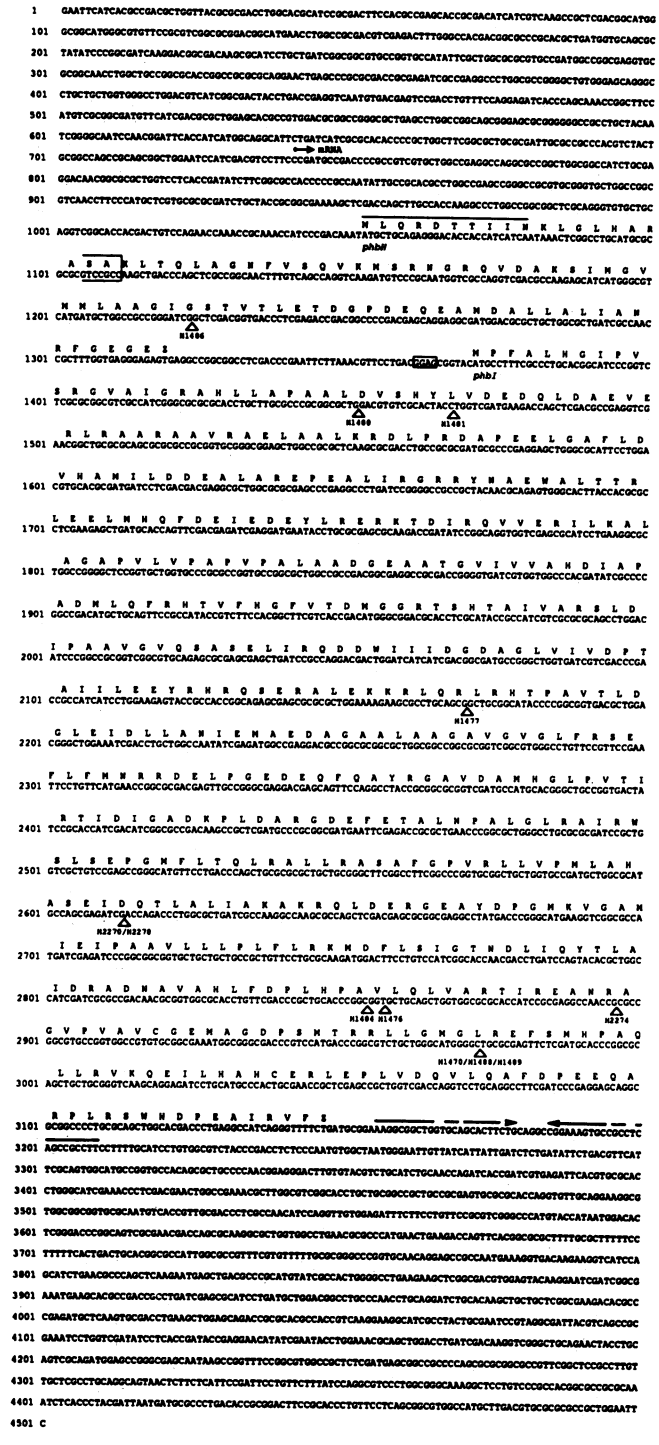

FIG. 3. Nucleotide sequence of the region in the genome of *A. eutrophus* relevant for the expression of the PHB-leaky phenotype. Amino acids deduced from the nucleotide sequence of the tentative genes are specified by standard one-letter abbreviations. The amino acid sequence of protein H, which was confirmed by sequence analysis of the N terminus of the *phbH'-'lacZ* fusion gene product, is overlined. The 3' region of the *phbH'* portion of the *phbH'-'lacZ* fusion is indicated by a bracket. A putative ribosome-binding site is boxed. The position of a possible hairpinlike structure downstream of *phbI* is indicated by inverted arrows. The position of Tn*5* insertions in PHB-leaky mutant strains are indicated by triangles with the designation of the strain.

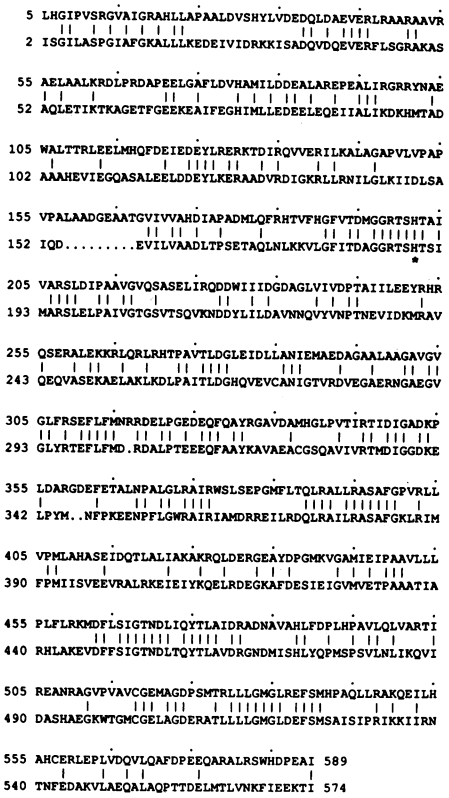

FIG. 4. Alignment of the deduced amino acid sequences of protein I deduced from the *A. eutrophus phbI* gene (upper line) and of the enzyme I protein (11) of the *E. coli* PTS (bottom line). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate the positions of the amino acids within the protein. Vertical lines between amino acids indicate identity at that position. The position of His-189 of the *E. coli* enzyme I is indicated by an asterisk.

all transposon insertions (see below and Fig. 2 and 3). It covered ORF1 completely but was oriented in the opposite direction.

Structure of the putative gene produce of ORF1. The amino acid sequence deduced from the nucleotide sequence of ORF1 was compared with the primary structures of other proteins. It exhibited homology to the enzyme I of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PEP-PTS) from various sources. With protein EI of the *E. coli* PEP-PTS, for instance, an identity of 38.9% and a similarity of 61.3% of the amino acids were obtained (Fig. 4). Furthermore, an alignment of amino acids 5 to 311 or 4 to 105 of the putative translation product of ORF1 with amino acid residues 2 to 299 and 3 to 102, respectively, of the *ptsI* gene products of *Salmonella typhimurium* (6) or *Bacillus subtilis* (21) revealed 38.6 and 28.0% identical amino acid residues, respectively. The alignment of amino acid residues 168 to 543 of the tentative protein I of *A. eutrophus* with amino acids 424 to 790 of the *Rhodobacter capsulatus* MTP protein (67) gave the highest percent identity, 42.4%.

The ATG at nucleotide 1371 in Fig. 3 is most probably the translational initiation codon of the structural gene which we designate as *phbI*. This was concluded from the alignment of the amino acid sequences shown in Fig. 4 and from the tentative ribosome-binding site which preceded this putative

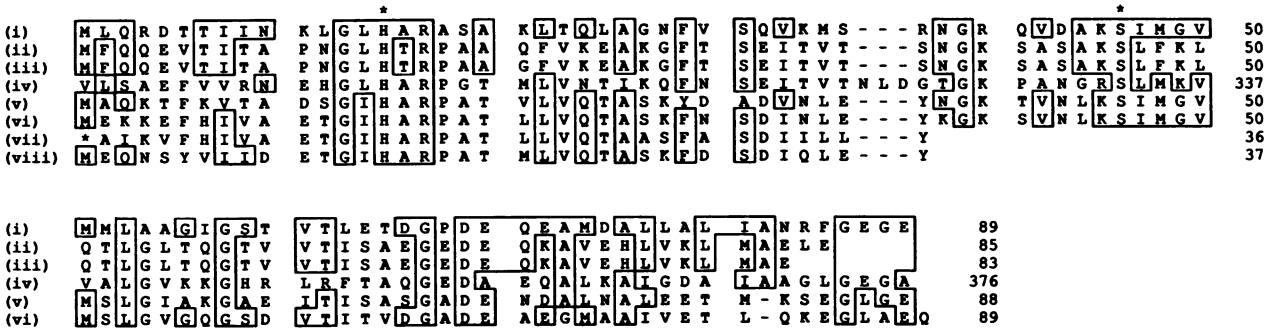

FIG. 5. Alignment of the deduced amino acid sequences of the *A. eutrophus phbH* gene product and the HPr proteins of the PTS from various sources. (i) *A. eutrophus phbH* gene product (this study); (ii) *E. coli ptsH* gene product (11); (iii) *S. typhimurium ptsH* gene product (41); (iv) *S. typhimurium fru* gene product (19, 20); (v) *B. subtilis ptsH* gene product (21); (vi) *S. faecalis* HPr (12); (vii) *S. sanguis* HPr (31); and (viii) *S. aureus* HPr (31). Amino acids are specified by standard one-letter abbreviations. Amino acid residues, which are identical with *A. eutrophus* protein H at one particular position, are boxed. Asterisks indicate the positions of the regulatory seryl residue (Ser-46 [13]) and of the catalytic histidyl residue (His-15 [18]), which are subject to phosphorylation-dephosphorylation.

translational start codon. For protein I, which was encoded by *phbI*, an M_r of 65,167 was calculated.

Structure of the putative gene product of ORF2. Computer-assisted alignments of the amino acid sequence deduced from the nucleotide sequence of ORF2, which started with the ATG at position 743, with the primary structures of proteins from data banks revealed very weak homology to the HPr protein of the *E. coli* PEP-PTS. The degree of homology was drastically enhanced if only the primary structure of the protein translated from the second ATG codon at nucleotide 1052 was included in the analysis. The primary structure of the deduced protein exhibited identities of 34.1, 34.9, 36.4, 39.3, 27.8, and 35.2% with the primary structures of the HPrs from *E. coli*, *S. typhimurium*, *B. subtilis*, *Streptococcus faecalis*, *Streptococcus sanguis*, and *Staphylococcus aureus*, respectively. With the *S. typhimurium* FPr protein, an identity of 27.2% was obtained (Fig. 5).

Recently, the PHB synthase structural gene (*phbC*) was fused upstream of the *lacZ'* gene of the promoter probe vector pMC1403 (42). Analysis of the *phbC'-'lacZ* gene fusion revealed the translational start site of the *phbC* transcript (54). Therefore, a fusion gene was constructed which consisted of the 5' region of ORF2 and of the *E. coli lacZ'* gene. The 5.5-kb *Apa*I-*Bst*EII subfragment (AB5500) of fragment PE1 was treated with *Bal* 31, and the deleted fragments were ligated into the *Sma*I site of the promoter probe vector pMC1403. The resulting plasmid was referred to as pHZ1. From this an 8.8-kb *Sal*I-fragment harboring the ORF2'-'*lacZ* fusion was cloned into the *Sal*I site of the broad-host-range vector pVK101. The resulting plasmid, which was referred to as pHZ2, was conjugationally transferred to *A. eutrophus*. It harbored 123 or 20 codons of the 5' region of ORF2 depending on whether the first or second ATG codon was considered a translational start codon. By affinity chromatography on APTG-Sepharose, a fusion protein with an M_r of approximately 118,000 was enriched from gluconate-grown cells of *A. eutrophus* harboring plasmid pHZ2 which were harvested in the stationary growth phase. The protein was transferred to a polyvinylidene difluoride membrane from a sodium dodecyl sulfate-polyacrylamide gel. The area of the membrane which harbored the gene fusion product was cut off. The N-terminal amino acid sequence, which was determined by automated Edman degradation, confirmed translation initiation from the second start codon (Fig. 3).

The structural gene, which was referred to as *phbH* and which encoded protein H with an M_r of 9,469, was not preceded by a typical Shine-Dalgarno sequence. As already indicated during the analysis of the fusion gene and the isolation of the fusion gene product (not shown in detail), this is consistent with only weak expression of *phbH'-'lacZ* gene fusion in *A. eutrophus*; in *E. coli*, expression did not occur at all.

Codon usage. For the region which has been sequenced in this study, a G+C content of 66.9 mol% was determined, which is close to the G+C content determined for the total genomic DNA of this bacterium (66.3 to 66.9 mol%). The G+C contents of ORF1 and ORF2 were 64.9 and 68.8 mol%, respectively. A high bias for codons with either G or C at the third position occurred for ORF1 (88.9 mol%), ORF2 (82 mol%), and *phbH* (91.3 mol%). According to Bibb et al. (2), the theoretical value is 84.5 mol%. In contrast, the G+C contents at the second position were 43.3 mol% (ORF1), 66.0 mol% (ORF2), 41.6 mol% (*phbH*), and 46.0 mol% (theoretical value); the G+C contents at the first position were 62.2 mol% (ORF1), 55.5 mol% (ORF2), 73.4 mol% (*phbH*), and 67.0 mol% (theoretical value). The codon usage of ORF3 (Table 3) did not obey the rules of Bibb et al. (2), and the G+C contents were 89.2, 46.3, and 69.4 mol% for the first, second, and third codon positions, respectively. This and the analysis of the *phbH'-'lacZ* gene fusion product (see below) clearly indicated that ORF3 does not represent a structural gene. In addition, ORF3 was not preceded by a Shine-Dalgarno sequence.

Putative transcriptional start sites. By S1 nuclease protection assays, which employed total RNA isolated from gluconate-grown cells of the stationary growth phase and two different oligonucleotides for the annealing reaction (see Materials and Methods), a transcriptional start site was located at nucleotide 740, which is 311 bp upstream of *phbH* (Fig. 6). No σ^{70} consensus promoter sequence was identified upstream of this putative transcriptional start site or at any other position upstream of *phbH*. Three regions, which exhibited weak homology (10, 8, and 9 of 13 possible matches) to the enterobacterial σ^{54} promoter consensus sequence (43), were located approximately 60, 280, and 370 nucleotides, respectively, upstream of *phbH*. They are probably not relevant to the expression of *phbH* and probably appear accidentally as a result of the high G+C content of

TABLE 3. Codon usage in *phbH*, *phbI*, *phbC*, and ORF3

Amino acid	Codon	Usage in gene:			
		phbH	*phbI*	*phbC*	ORF3
Ala	GCU	0	2	2	8
	GCA	0	4	6	6
	GCC	9	39	41	31
	GCG	5	41	32	40
Arg	AGA	0	0	0	2
	CGA	0	0	1	9
	AGG	1	1	0	3
	CGU	0	2	1	8
	CGG	0	14	1	31
	CGC	4	36	29	42
Asn	AAU	2	1	6	0
	AAC	2	6	19	1
Asp	GAU	0	11	7	34
	GAC	5	33	24	17
Cys	UGU	0	0	0	0
	UGC	0	2	5	7
Gln	CAA	0	0	3	2
	CAG	5	19	19	69
Glu	GAA	0	11	9	20
	GAG	5	35	20	9
Gly	GGA	1	1	1	5
	GGU	2	0	4	18
	GGG	1	7	4	8
	GGC	5	28	40	42
His	CAU	1	5	6	17
	CAC	0	14	8	26
Ile	AUA	0	0	0	2
	AUU	0	0	2	5
	AUC	5	34	21	11
Leu	UUG	0	1	4	0
	UUA	0	0	0	1
	CUU	0	2	2	13
	CUA	0	0	0	0
	CUC	3	7	5	38
	CUG	7	67	46	21
Lys	AAA	1	0	3	1
	AAG	3	11	18	4
Met	AUG	6	17	8	6
Phe	UUU	2	0	4	0
	UUC	0	19	16	11
Pro	CCA	0	0	4	7
	CCU	0	2	1	4
	CCG	0	25	26	16
	CCC	1	6	4	8
Ser	AGU	0	0	0	1
	UCU	0	0	0	1
	UCA	0	0	1	4
	UCC	2	4	4	4
	UCG	1	16	13	2
	AGC	2	6	9	6
Thr	ACA	0	0	2	2
	ACU	0	1	2	1
	ACG	1	3	8	2
	ACC	5	16	21	11
Trp	UGG	0	4	19	1
Tyr	UAU	0	1	5	0
	UAC	0	6	14	0
Val	GUA	0	0	1	8
	GUU	0	1	0	6
	GUC	4	16	14	34
	GUG	1	24	24	14

the genomic DNA. No promoter consensus sequences were detected upstream of *phbI*.

Putative transcriptional termination sites. Immediately downstream of *phbI* an inverted repeat was localized which may represent a factor-dependent transcriptional terminator (Fig. 3). The free energy of this structure is approximately 132 kJ/mol. No comparable structures were detected downstream of *phbH*. There was also no evidence for the presence of the *E. coli crr*-like gene (11) downstream of *phbI*.

Mapping of Tn*5* insertions. From pHC79 hybrid cosmids harboring the *Eco*RI restriction fragments of Tn*5*-induced mutants, which were complemented by plasmid pBL7502 or pBH4550, the *Sal*I subfragments, which conferred kanamycin resistance, were cloned in pBluescript SK. By using a synthetic oligonucleotide (5′-GTTAGGAGGTCACATGG-3′), which hybridized at a distance of 63 to 79 bp from the end of IS*50*L, for nucleotide sequencing, the exact locations of Tn*5* in each of the mutant fragments mentioned above were identified (Fig. 2 and 3). In mutant strain H1486 the insertion of Tn*5*::*mob* was localized in *phbH*. In contrast, insertions were mapped in *phbI* in mutant strains H1470, H1476, H1477, H1480, H1481, H1484, H1488, H1489, H2270, H2274, and H2278 (Fig. 3). In mutant strains H1470, H1488, and H1489, insertions of Tn*5*::*mob* were localized at identical positions; these mutants derived from the same mating and are probably parallel strains. Mutant strains H2270 and H2278 also resulted from the same mating.

Complementation of HPr mutants of *E. coli*. To test whether the cloned genes were able to complement PTS$^-$ mutants of *E. coli*, plasmids pBL7502 and pBH4550 were each transformed to *E. coli* L191. The transformants were grown on MacConkey agar plates with fructose or glucose (1.0% [wt/vol]). On fructose as well as on glucose, the transformants formed white colonies, indicating that they were unable to use these carbohydrates. As the *phbH′-′lacZ* gene fusion was probably not expressed in *E. coli* (see above), it remains unclear whether *phbH* and *phbI* can replace the physiological function of the PEP-PTS genes from *E. coli*.

DISCUSSION

The analysis of the gene loci *phbH* and *phbI*, which are inactivated in Tn*5*-induced PHB-leaky mutants of *A. eutrophus*, revealed the presence of two proteins which were referred to as protein H and protein I. These proteins exhibit

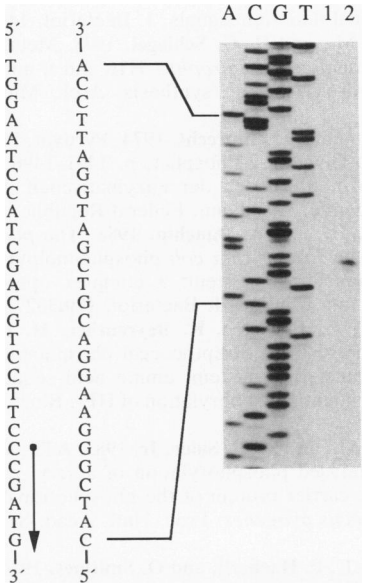

FIG. 6. S1 nuclease protection assay of the transcript of *phbH*. Lanes: A, C, G, and T, standard sequencing reactions to size the mapping signals; 1, RNA from *A. eutrophus* H16; 2, RNA from *A. eutrophus* H16(pHZ2). RNA was harvested from gluconate-grown cells in the stationary growth phase. The origin and direction of transcription are indicated by an arrow.

striking sequence similarity with HPr and enzyme I, respectively, which are components of the PEP-PTS present in various bacteria. The primary structure of protein H shows homology around the active-site His residue (His-15 and Arg-17) of known HPrs, and also around Ser-46, which is phosphorylated in the HPrs of gram-positive but not of gram-negative bacteria (53). In general, some regions of protein H from *A. eutrophus* are more similar to the HPrs of gram-negative bacteria, whereas other regions are more similar to regions of HPrs of gram-positive bacteria. However, marked differences occurred at highly conserved amino acids: Pro-18, Ala-19, Val-23, Gly-54, and Ile-63 were replaced by Ala, Ser, Thr, Ala, and Leu, respectively, in protein H.

Although *phbH* is not preceded by a typical Shine-Dalgarno sequence, (i) the synthesis of the *phbH'-'lacZ* fusion protein, (ii) the complementation studies, and (iii) the phenotype of mutant strains H1486 clearly indicated that protein H is also essential for maximal accumulation of PHB in *A. eutrophus*. The sequence data indicate that *phbH* and *phbI* are organized as a single transcription unit (*phbHI*), as are the corresponding genes from *E. coli* (11). That transcription may occur only at a very low rate is indicated by the occurrence of only weak signals in S1 nuclease protection assays and by the lack of typical *E. coli* promoter consensus sequences upstream of the transcriptional start site. That plasmid pBL7502 allows complementation of PHB-leaky mutants, which harbor Tn*5* in *phbI*, indicates that λ DNA, which is localized upstream of the *Eco*RI site in this plasmid, provides some promoter activity. A low transcription rate is consistent with a putative regulatory function of both proteins (see below). It has yet to be determined whether the inability to complement *E. coli pts* mutants results from weak or even absent expression of *phbHI* in *E. coli* or whether protein H and protein I cannot functionally replace the corresponding components of the *E. coli* PEP-PTS.

Since physiological studies had not indicated that the uptake of carbon sources was affected in PHB-leaky mutants, and since these mutants did not exhibit any pleiotropic effect, it is unlikely that protein H and protein I participate in transport processes in *A. eutrophus* H16. The functions of the *phbH* and *phbI* gene products are still unknown and have yet to be evaluated. In contrast, evidence for the presence of PEP-PTS had been obtained in some marine, unrelated *Alcaligenes* species (47). From the observation that the PHB content in cells of PHB-leaky mutants decreases more rapidly than in cells of the wild type after the extracellular carbon source has been exhausted, the following speculative hypothesis for the function of the HPr-like and enzyme I-like proteins in *A. eutrophus* is proposed (Fig. 7). Presumably both proteins have only a regulatory function and are involved in the regulation of the mobilization of PHB in the cells. Regulation may be achieved by phosphorylation in two different ways. (i) Cook and Schlegel (9) have provided

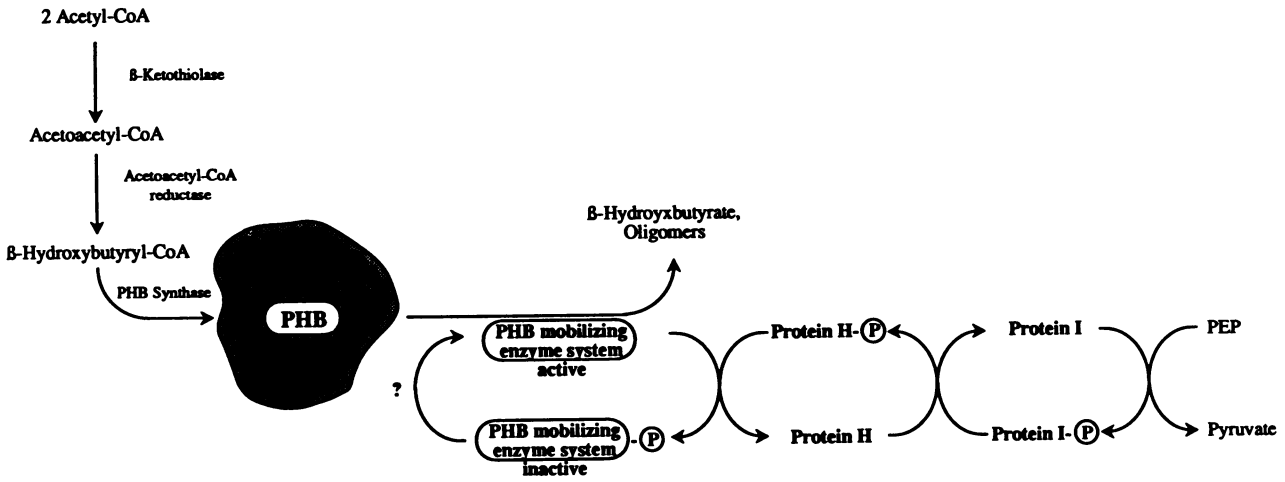

FIG. 7. Working hypothesis for the function of protein H and protein I in *A. eutrophus*. CoA, coenzyme A. The circled P represents phosphate.

evidence that the intracellular concentration of PEP is higher in the presence of an extracellular carbon source. If protein I accepts a phosphoryl group from PEP and transfers this group via protein H to a central component of the PHB-mobilizing enzyme system of *A. eutrophus*, this component will be inactivated (Fig. 7). (ii) Alternatively, regulation of PHB mobilization by PEP-PTS homologous proteins may be achieved at the transcriptional level. Proteins which affect the transcription of genes relevant for the PHB metabolism may be covalently modified by phosphorylation-dephosphorylation, conferring, e.g., different affinities to specific binding sites at the relevant gene loci. Examples are the *bglG* and *sacB* gene products in *E. coli* (51) and *B. subtilis* (52), respectively. In both procedures, the provision of cells with an extracellular carbon source may be sensed and signaled either directly or indirectly to the PHB-mobilizing enzyme system. Our hypothesis is physiologically sound and explains all data available for PHB-leaky mutants of *A. eutrophus*. Evidence for a different specific role, e.g., in chemotactic signaling, in addition to the transport function was obtained for the *ptsH* gene product in *E. coli* (22). If these hypotheses hold true, additional compounds are probably involved in the regulation of the PHB-mobilizing enzyme system in *A. eutrophus*. This is indicated by the occurrence of PHB-leaky mutants (Table 2, class C), which are not complemented by the *phbH* or *phbI* genes. Further studies are necessary to evaluate this very intriguing aspects of PHB metabolism.

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