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Accumulation of the PhaP Phasin of *Ralstonia eutropha* Is Dependent on Production of Polyhydroxybutyrate in Cells

GREGORY M. YORK,[1] BJÖRN H. JUNKER,[1]† JOANNE STUBBE,[1,2] AND ANTHONY J. SINSKEY[1]*

Department of Biology[1] and Department of Chemistry,[2] Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Polyhydroxyalkanoates (PHAs) are polyoxoesters that are produced by diverse bacteria and that accumulate as intracellular granules. Phasins are granule-associated proteins that accumulate to high levels in strains that are producing PHAs. The accumulation of phasins has been proposed to be dependent on PHA production, a model which is now rigorously tested for the phasin PhaP of *Ralstonia eutropha*. *R. eutropha phaC* PHA synthase and *phaP* phasin gene replacement strains were constructed. The strains were engineered to express heterologous and/or mutant PHA synthase alleles and a *phaP-gfp* translational fusion in place of the wild-type alleles of *phaC* and *phaP*. The strains were analyzed with respect to production of polyhydroxybutyrate (PHB), accumulation of PhaP, and expression of the *phaP-gfp* fusion. The results suggest that accumulation of PhaP is strictly dependent on the genetic capacity of strains to produce PHB, that PhaP accumulation is regulated at the level of both PhaP synthesis and PhaP degradation, and that, within mixed populations of cells, PhaP accumulation within cells of a given strain is not influenced by PHB production in cells of other strains. Interestingly, either the synthesis of PHB or the presence of relatively large amounts of PHB in cells (>50% of cell dry weight) is sufficient to enable PhaP synthesis. The results suggest that *R. eutropha* has evolved a regulatory mechanism that can detect the synthesis and presence of PHB in cells and that PhaP expression can be used as a marker for the production of PHB in individual cells.

Polyhydroxyalkanoates (PHAs) are polyoxoesters that are produced by diverse bacteria as intracellular storage compounds and that can be used to make biodegradable plastics (7, 14, 16, 21, 26). PHA synthases and phasins are proteins that play important roles in PHA production. PHA synthases play the central catalytic role in PHA synthesis and granule formation by catalyzing the polymerization of hydroxyacyl coenzyme A substrates to yield PHAs (9), which in turn associate to form PHA granules (8, 16). Studies on the PHA synthases of *Ralstonia eutropha* ($PhaC_{Re}$) and *Chromatium vinosum* ($PhaEC_{Cv}$) have yielded important insights on the mechanism of PHA synthesis, namely, that a cysteine residue conserved among these two proteins ($PhaC_{Re}$ C319 and $PhaC_{Cv}$ C149) and among all known PHA synthases is involved in covalent catalysis (9, 20, 36) and that the synthases share structural and functional similarities with lipases (11, 12, 15). Phasins, on the other hand, play a poorly understood role in PHA synthesis and granule formation (32, 37). Phasins are low-molecular-weight proteins, designated PhaP, that share no sequence homology and have been identified from many bacterial strains based on their accumulation to high levels in cells producing PHAs and their association with PHA granules (17, 19, 23, 35). Phasins from several bacterial strains have been shown to increase production of PHAs and to promote the accumulation of PHAs as numerous small granules in cells (17, 35, 37). These two effects are likely related, but the precise role played by phasins remains to be determined. Efforts to understand the regulation and function of phasins are the major focus of the study reported here.

Several lines of evidence suggest that regulation of phasin accumulation is important for phasin function and that accumulation of phasins is tightly coupled to PHA synthesis. Studies with an *R. eutropha phaP* deletion strain and several strains expressing low levels of PhaP indicate that these strains exhibit a 50% decrease in PHA production relative to the wild-type (wt) strain (37), suggesting that phasin must accumulate to high levels in order to promote PHA synthesis. Studies of *phaC*::Tn*5* and spontaneous PHA-null mutants of *R. eutropha* suggest that PhaP accumulation requires PHA synthesis (35), and studies of several spontaneous PHA-leaky mutants of *Rhodococcus ruber* suggest that phasin levels generally match PHA levels (23). These latter two studies, however, leave open the possibility that factors such as the physical absence of $PhaC_{Re}$ from cells, effects on expression of genes downstream of *phaC*, or defects in cell growth, rather than defects in PHA production, are actually responsible for defects in phasin accumulation. These studies are also ambiguous with regard to whether PhaP accumulation is regulated at the level of PhaP synthesis and/or degradation and whether PhaP accumulation is regulated at the level of individual cells or populations of cells. Recent studies of the PhaF protein of *Pseudomonas oleovorans* and the PhaR protein of *Paracoccus denitrificans* provide useful insights into how the expression of proteins involved in PHA synthesis, including phasins, may be negatively regulated in the absence of PHA (17, 24). Specifically, PhaF has been proposed to function as a negative regulator of transcription that can be titrated from DNA by PHA (24), and PhaR may function similarly (17). The generality of PhaF and

* Corresponding author. Mailing address: Bldg. 68-370, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139. Phone: (617) 253-6721. Fax: (617) 253-8550. E-mail: asinskey@mit.edu.

† Present address: Max-Planck-Institute of Molecular Plant Physiology, 14476 Golm, Germany.

TABLE 1. Strains and plasmids used in this study[a]

Strain or plasmid	Description[b]	Reference or source
R. eutropha strains		
AeH16	wt, Gm resistant, also termed DSM 428 and ATCC 17699	ATCC 17699
Re1000	*phaC3*::Tn*5*, blocked in PHB synthesis due to Tn*5* insertion in *phaC* ORF	22
Re1001	*phaP-gfp* translational-fusion gene replacement strain, derived from Ae H16/pGY15	This study
Re1007	*phaP-gfp* translational-fusion gene replacement strain, derived from *phaC3*::Tn*5*/pGY19	This study
Re1017	*phaC* partial-deletion gene replacement strain, derived from Ae H16/pGY47	This study
Re1022	$phaC_{Re}$ C319A gene replacement strain, derived from Re1017/pGY31	This study
Re1031	$phaEC_{Cv}$ gene replacement strain, derived from Re1034/pGY53	This study
Re1034	*phaC* precise-deletion gene replacement strain, derived from Ae H16/pGY46	This study
Re1036	$phaC_{Cv}$ gene replacement strain, derived from Re1034/pGY52	This study
Re1052	*phaP* precise-deletion gene replacement strain, derived from Ae H16/pGY63	37
Re1058	$phaEC_{Cv}$ C149A gene replacement strain, derived from Ae H16/pGY67	This study
E. coli strains		
DH5α	Strain for ligation/cloning experiments	NEB[c]
S17-1	Strain for conjugative transfer of plasmids into *R. eutropha*	30
Plasmids		
pBluescript II KS	High-copy-number plasmid used for cloning, confers Ap resistance	Stratagene
pGY1a+	*phaP-gfp* translational-fusion cloned into pSW213, useful for expression of GFP in *E. coli*	This study
pGY15	*phaP-gfp* translational-fusion gene replacement plasmid, confers Km resistance	This study
pGY19	*phaP-gfp* translational-fusion gene replacement plasmid, confers Sm/Sp resistance	This study
pGY31	$phaC_{Re}$ C319A gene replacement plasmid, confers Km resistance	This study
pGY46	*phaC* precise-deletion gene replacement plasmid, confers Km resistance	This study
pGY47	*phaC* partial-deletion gene replacement plasmid, confers Km resistance	This study
pGY52	$phaC_{Cv}$ gene replacement plasmid, confers Km resistance	This study
pGY53	$phaEC_{Cv}$ gene replacement plasmid, confers Km resistance	This study
pGY63	*phaP* precise-deletion gene replacement plasmid, confers Km resistance	37
pGY67	$phaEC_{Cv}$ C149A gene replacement plasmid, confers Km resistance	This study
pJQ200mp18	Gene replacement vector; encodes *sacB, oriV, oriT, traJ*; confers Gm resistance	25
pJQ200mp18Km	Derivative of pJQ200mp18, Gm resistance gene disrupted, confers Km resistance	37
pJQ200mp18SmSp	Derivative of pJQ200mp18, Gm resistance gene disrupted, confers Sm/Sp resistance	This study
pKAS4-C319A	$phaC_{Re}$ C319A allele cloned into plasmid pKAS4	9
pKENgfpmut2	*gfpmut2* allele cloned into multicloning site of pKEN1, confers Ap resistance	4, 6
pSW213	Low-copy-number plasmid, confers Tc resistance	3
pUC19	High-copy-number plasmid used for cloning, confers Ap resistance	NEB
pUM4-C149A	$phaEC_{Cv}$ C149A allele cloned into plasmid pUM4	20
pUT-miniTn5-Km	Source of Km resistance gene for pGY15	5
pUT-miniTn5-SmSp	Source of Sm/Sp resistance gene for pJQ200mp18SmSp	5

[a] Plasmids constructed in this study that were used only as intermediates for construction of other plasmids are described only in Materials and Methods.
[b] Abbreviations: Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.
[c] NEB, New England Biolabs.

PhaR-mediated regulation in PHA synthesis remains to be determined.

We are interested in developing a model for regulation of phasin accumulation. Recent advances in understanding of the mechanism of $PhaC_{Re}$ (9), combined with the fact that *R. eutropha* is readily amenable to genetic manipulation (22, 28, 31) and produces poly-[(*R*)-3-hydroxybutyrate] (PHB) under many standard cultivation conditions (16, 34), make *R. eutropha* an excellent organism in which to address this goal. Thus, we have constructed a set of *R. eutropha phaC* deletion and gene replacement strains and have analyzed these strains with respect to growth, PHB production, and PhaC and PhaP accumulation. An *R. eutropha* strain carrying a *phaP-gfp* translational fusion in place of the wt allele of PhaP was also constructed and analyzed with respect to expression of green fluorescent protein (GFP). The results suggest that accumulation of PhaP is strictly dependent on the genetic capacity of strains to produce PHB, that *R. eutropha* has evolved a regulatory mechanism that can detect the synthesis and presence of PHB in cells, and that PhaP expression can be used as a marker for the production of PHB in individual cells.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. The strains and plasmids used in this study are listed in Table 1. The oligonucleotides used in this study are listed in Table 2.

Growth media. *R. eutropha* strains were cultivated on one of the following media, depending on the particular application: Luria-Bertani (LB) medium (18), tryptic soy broth dextrose-free (TSB) medium (Becton Dickinson Microbiology Systems, Cockeysville, Md.), PHA(no carbon), PHA(med), PHA(high), or PHB production medium. PHA(no carbon), PHA(med), and PHA(high) are based on a minimal medium (22) supplemented with fructose (0, 0.5, and 1%, respectively) and ammonium chloride (0.5, 0.1, and 0.01%, respectively). PHB production medium is identical to PHA(high) except that it contains 40% less of the following components: fructose, ammonium chloride, and trace salts. *Escherichia coli* strains were cultivated on LB medium.

Antibiotics. Antibiotics were added to growth media to the following final concentrations: for *R. eutropha*, gentamicin (10 μg/ml), kanamycin (270 μg/ml), and spectinomycin (250 μg/ml); for *E. coli*, ampicillin (100 μg/ml), gentamicin (10 μg/ml), kanamycin (25 μg/ml), spectinomycin (100 μg/ml), and tetracycline (10 μg/ml).

Cultivation conditions. *R. eutropha* and *E. coli* strains were cultivated with aeration at 30 and 37°C, respectively. For the preparation of genomic DNA or selection for resistance to antibiotics, *R. eutropha* strains were cultivated in liquid TSB medium or solid LB agar (1.2%). For PHB production analyses, *R. eutropha* strains were cultivated in 4 ml of TSB in test tubes to saturation (24 to 30 h). Aliquots (1 ml) were transferred into 50 ml of TSB in 250-ml baffled flasks and

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Location and orientation[b]
gfp1	AATTC**CTCGAG**ATGAGTAAAGGAGAAGAACTTTTC	5′ end of *gfp* ORF (+)
gfp2	AGCTT**GGATCCGCATGCCTGCAG**GTCTGG	3′ end of *gfp* ORF (−)
phaC2	AGCTT**GGATCC**GATGCGAGCGCTGCATACC	5′ end of region upstream of *phaC* ORF (+)
phaC3	**P**–GATTTGATTGTCTCTCTGCCG	3′ end of region upstream of *phaC* ORF (−)
phaC4	**P**–CGCTTGCATGAGTGCCGGCG	5′ end of region downstream of *phaC* ORF (+)
phaC5	AGCTT**GGATCC**GGCGCTCATGTTTTCCTGG	3′ end of region downstream of *phaC* ORF (−)
phaC6	**P**–ATTGAGCAGGTAGAACGCGG	Internal to *phaC* ORF (−)
phaC7	**P**–ATCGCCGGTGTGATCAACCC	Internal to *phaC* ORF (+)
phaCcv1	**P**–ATGTTCCCCATCGACATCCG	5′ end of $phaC_{Cv}$ ORF (+)
phaCcv2	**P**–TTATCGCTCGTTGAGCCACTT	3′ end of $phaC_{Cv}$ ORF (−)
phaEcv1	**P**–ATGAGCAACACTAATTTCTTCAATG	5′ end of $phaEC_{Cv}$ ORF (+)
phaP2[c]	CCGA**GGATCC**ATCGCCGGACAAGGCAGC	Region downstream of *phaP* ORF (−)
phaP4	CTAGCGAATTC**GGATCC**GCAATCGCGCATCGTTG	5′ end of region upstream of *phaP* ORF (+)
phaP5	CTAGTGC**GTCGAC**CATTGCTGGTCTCCAGTGGTG	3′ end of region upstream of *phaP* ORF (−)

[a] Restriction sites engineered into sequences are indicated in boldface. P– indicates presence of 5′ phosphate group.
[b] Forward (+) or reverse (−) orientation relative to ORF is indicated.
[c] Residue underlined in *phaP2* corresponds to G in correct sequence of *phaP* but was designed as C based on an error in original published sequence. Despite the error, *phaP2* is functional in PCR amplification of the *phaP* gene.

cultivated for 12 h. Aliquots of washed cells were transferred into 200 ml of PHB production medium to yield cultures with an initial optical density at 600 nm (OD_{600}) of 1.0 and were cultivated for 72 h. For PHB utilization analyses, aliquots (50 ml) of washed cells were resuspended in 200 ml of PHA(no carbon) and were cultivated for an additional 72 h. For immunoblot analyses, *R. eutropha* strains were cultivated in 5 ml of TSB to saturation (approximately 36 h). Aliquots (100 μl) of culture were transferred into 5 ml of TSB and were cultivated to saturation (approximately 24 h). Aliquots of washed cells were transferred into 5 ml of TSB, PHA(med), and/or PHA(high) to yield cultures with an initial OD_{600} of 1.0 ($\sim 1 \times 10^9$ CFU/ml) and were cultivated for 48 h. For cocultivation experiments, *R. eutropha* strains were cultivated as described for immunoblot analyses, except that final cultures were inoculated with two strains, each added to an initial OD_{600} of 0.5 ($\sim 5 \times 10^8$ CFU of each strain/ml).

DNA preparation and manipulation. Standard approaches were used for preparation and manipulation of DNA and for the PCR (1). Genomic DNA was prepared from *R. eutropha* strains by the hexadecyltrimethyl ammonium bromide method (1) with one important modification: DNA was prepared from 250 μl of culture (rather than 1.5 ml of culture) without proportional adjustment of reagents. All constructs containing PCR products were confirmed by sequencing at the Massachusetts Institute of Technology Biopolymer Lab.

Construction of *phaC* precise-deletion (and *phaC* partial-deletion) gene replacement plasmid pGY46 (and pGY47). A 0.41-kb (or 0.78-kb) fragment of *R. eutropha* DNA, corresponding to the region immediately upstream of the $phaC_{Re}$ open reading frame (ORF) (or this region plus part of the $phaC_{Re}$ ORF), was amplified by PCR with the oligonucleotides phaC2 and phaC3 (or phaC2 and phaC6) such that a *Bam*HI site was introduced at the upstream end of the PCR product. A 0.45-kb (or 0.69-kb) fragment of *R. eutropha* DNA, corresponding to the region immediately downstream of the $phaC_{Re}$ ORF (or this region plus part of the $phaC_{Re}$ ORF), was amplified by PCR with the oligonucleotides phaC4 and phaC5 (or phaC7 and phaC5) such that a *Bam*HI site was introduced at the downstream end of the PCR product. A three-way ligation was conducted between the two PCR products and the vector pBluescript II KS, each of which had been digested with *Bam*HI and gel purified. The product, designated pGY26 (or pGY27), contains a 0.86-kb (or 1.47-kb) *Bam*HI fragment corresponding to a fusion of the regions upstream and downstream of $phaC_{Re}$ (or these regions plus part of the $phaC_{Re}$ ORF), cloned into the *Bam*HI site of pBluescript II KS. The 0.86-kb (or 1.47-kb) *Bam*HI fragment from pGY26 (or pGY27) was cloned into the *Bam*HI site of pJQ200mp18Km to yield pGY46 (or pGY47). Note that the *phaC* partial deletion gene replacement plasmid pGY47 and the resulting *R. eutropha* strain Re1017 were used as intermediates for construction of the $phaC_{Re}$ C319A mutant strain and were not studied further.

Construction of $phaC_{Cv}$ (and $phaEC_{Cv}$) gene replacement plasmid pGY52 (and pGY53). A 1.1-kb (or 2.2-kb) fragment of *C. vinosum* DNA, corresponding to the $phaC_{Cv}$ ORF (or $phaEC_{Cv}$ ORFs), was amplified by PCR with oligonucleotides phaCcv1 and phaCcv2 (or phaECcv1 and phaCcv2). The 1.1-kb $phaC_{Cv}$ (or 2.2-kb $phaEC_{Cv}$) PCR fragment was digested with *Aat*II to yield a 0.30-kb (or 1.43-kb) N-terminal fragment and a 0.77-kb C-terminal fragment. A 0.41-kb fragment of *R. eutropha* DNA, corresponding to the region immediately upstream of the $phaC_{Re}$ ORF, was amplified by PCR with oligonucleotides phaC2 and phaC3 such that a *Bam*HI site was introduced at the upstream end of the PCR product. This fragment was digested with *Bam*HI. A 0.45-kb fragment of *R. eutropha* DNA, corresponding to the region immediately downstream of the $phaC_{Re}$ ORF, was amplified by PCR with oligonucleotides phaC4 and phaC5 such that a *Bam*HI site was introduced at the downstream end of the PCR product. This fragment was digested with *Bam*HI. A three-way ligation was conducted with the 0.41-kb *Bam*HI-blunt upstream fragment, the 0.30-kb (or 1.43-kb) blunt-*Aat*II N-terminal fragment, and the 2.2-kb *Bam*HI-*Aat*II fragment of pUC19, to yield pGY49 (or pGY36), in which the region upstream of the $phaC_{Re}$ ORF is cloned immediately upstream of, and in the same orientation as, the 0.30-kb (or 1.43-kb) N-terminal end of the $phaC_{Cv}$ ORF (or $phaEC_{Cv}$ ORFs). A three-way ligation was conducted with the 0.77-kb *Aat*II-blunt C-terminal fragment, the 0.45-kb blunt-*Bam*HI downstream fragment, and the 2.2-kb *Bam*HI-*Aat*II fragment of pUC19, to yield pGY48, in which the region downstream of the $phaC_{Re}$ ORF is cloned immediately downstream of, and in the same orientation as, the 0.77-kb C-terminal end of the $phaC_{Cv}$ ORF. A contiguous fragment of DNA ($phaC_{Re}$ upstream-$phaC_{Cv}$ ORF [or $phaEC_{Cv}$ ORFs]-$phaC_{Re}$ downstream) was constructed by three-way ligation of pBluescript II KS (digested with *Bam*HI), the 0.7-kb (or 1.84-kb) *Bam*HI/*Aat*II fragment of pGY49 (or pGY36), and the 1.2 kb *Aat*II/*Bam*HI fragment of pGY48, to yield pGY50 (or pGY51). The 1.9-kb (or 3.0-kb) *Bam*HI fragment of pGY50 (or pGY51) was cloned into the *Bam*HI site of pJQ200mp18Km to yield pGY52 (or pGY53).

Construction of $phaC_{Re}$ C319A gene replacement plasmid pGY31. A 1.8-kb *Eco*RI/*Bam*HI fragment corresponding to the entire $phaC_{Re}$ C319A ORF was isolated from pKAS4-C319A, treated with Klenow to generate blunt ends, and cloned into the *Sma*I site in the multicloning site of pJQ200mp18Km to yield pGY31.

Construction of $phaEC_{Cv}$ C149A gene replacement plasmid pGY67. A 0.72-kb *Asc*I-*Dra*III fragment of pUM4-C149A, including the $phaC_{Cv}$ C149A mutation, was used to replace the corresponding fragment of pGY53 in order to yield pGY67.

Construction of gene replacement vector pJQ200mp18SmSp. pJQ200mp18SmSp was constructed by cloning the 2-kb *Bam*HI fragment encoding streptomycin/spectinomycin resistance from pUT-miniTn*5*-SmSp into the *Bgl*II site within the gentamicin resistance gene of pJQ200mp18. pJQ200mp18SmSp can be used to select for maintenance of plasmids in *R. eutropha* strains carrying Tn*5* insertions, given that it encodes spectinomycin resistance.

Construction of *phaP-gfp* translational-fusion gene replacement plasmids pGY15 and pGY19 and GFP expression plasmid pGY1a+. A 0.77-kb fragment of *R. eutropha* DNA, corresponding to the region immediately upstream of the *phaP* ORF, was amplified by PCR (oligonucleotides, phaP4 and phaP5) such that a *Bam*HI site and a *Sal*I site were introduced at the upstream and downstream ends, respectively. The PCR product was cloned into the *Eco*RV site of pBluescript II KS, yielding pphaP2. A 0.75-kb fragment of pKENgfpmut2, corresponding to the *gfp* ORF, was amplified by PCR (oligonucleotides, gfp1 and gfp2) such that an *Xho*I site and a *Bam*HI site were introduced at the upstream and downstream ends, respectively. The PCR product was cloned into the *Eco*RV site of pBluescript II KS, yielding pgfp2. The 0.77-kb *Bam*HI-*Sal*I fragment of pphaP2 and the 0.75-kb *Xho*I-*Sal*I fragment of pgfp2 were excised from their

respective plasmids, ligated, and treated with *Bam*HI, *Sal*I, and *Xho*I. The resulting 1.5-kb ligation product was cloned into the *Bam*HI site of pSW213, yielding pGY1a+, and was also cloned into the *Bam*HI site of pBluescript II KS, yielding pKSphaPgfp7. A 1.5-kb fragment of *R. eutropha* DNA, corresponding to the region upstream of the *phaP* gene and the entire *phaP* ORF, was amplified by PCR (oligonucleotides, phaP4 and phaP2) such that *Bam*HI sites were introduced at both ends of the PCR product. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of the vector pSW213 to yield pGY4+. A 1.6-kb *Hin*dIII-*Pst*I (partial digest) fragment of pKSphaPgfp7 was cloned into pGY4+, which had been digested by *Hin*dIII and *Pst*I. The resulting plasmid, pGY11a, contains the *phaP* promoter-*gfp* ORF fusion adjacent to a truncated version of the *phaP* ORF. The 2.1-kb *Bam*HI fragment of pGY11a was cloned into the *Bam*HI site of pJQ200mp18 to yield pGY12. pGY15 was constructed by cloning the 2.0-kb *Bam*HI fragment encoding kanamycin resistance from pUTminiTn*5*-Km into the *Bgl*II site within the gentamicin resistance gene of pGY12. pGY19 was constructed by cloning the 2.14-kb *Bam*HI fragment of pGY11a into the *Bam*HI site of pJQ200mp18SmSp.

Construction of *phaC* and *phaP* gene replacement strains. Gene replacement was accomplished by adaptation of standard protocols (25, 31). The combinations of starting strains and plasmids used for construction of each gene replacement strain are indicated in Table 1. Each gene replacement construction was designed and carried out such that a successful gene replacement strain could be distinguished from the starting strain based on the size of the *phaC* or *phaP* allele in the chromosome, as determined by PCR. Successful gene replacement strains were identified and confirmed based on PCR analyses and Southern blot analyses.

Quantitation of PHB in *R. eutropha* cells. PHB was quantitated by the sulfuric acid/high-pressure liquid chromatography method of Karr et al. (13) (column, Aminex HPX 87H [Bio-Rad, Hercules, Calif.]; column temperature, 50°C; gradient, isocratic; mobile phase, 0.014 N sulfuric acid; flow rate, 0.7 ml/min; detection system, UV detector, 210 nm). Samples corresponded to cells from 5 or 10 ml of culture that had been dried and weighed.

Preparation and quantitation of PhaC$_{Re}$, PhaEC$_{Cv}$, and PhaP proteins. PhaC$_{Re}$ (expressed as an N-terminal histidine tag fusion protein), PhaEC$_{Cv}$, and PhaP were purified as previously described (12, 20, 37). Experimentally determined extinction coefficients were used for quantitation of each protein (11, 12, 37).

Polyclonal antibodies against PhaC$_{Re}$, PhaEC$_{Cv}$, PhaP, and GFP. Rabbit polyclonal antibodies against PhaC$_{Re}$ and PhaP were generated by use of standard protocols (10) at Covance Antisera Services (Denver, Pa.). Preparation of antibodies against PhaEC$_{Cv}$ (20) has been described previously. Rabbit serum was filtered (0.45-μm-pore-size filter) prior to use. Antibodies against PhaP were further purified by binding and elution from Hi-Trap NHS (*N*-hydroxysuccinimide)-activated resin (Amersham Pharmacia Biotech, Piscataway, N.J.) to which PhaP had been cross-linked and by binding and elution from Hi-Trap protein G resin (Amersham Pharmacia Biotech), in both cases according to the manufacturer's instructions. Anti-GFP antibodies were obtained from Clontech (Palo Alto, Calif.).

Immunoblot analyses. Whole bacterial cells or purified protein samples were separated by sodium dodecyl sulfate–10 or 15% polyacrylamide gel electrophoresis (SDS–10 or 15% PAGE) (1). Bacterial cell samples corresponded to 10-μl aliquots of cells resuspended to an OD_{600} of 1.0. Proteins were transferred to Immobilon P polyvinyl difluoride membrane (Millipore, Bedford, Mass.) by electroblotting at 100 V for 1.5 h at ~4°C. Protein detection was accomplished by use of the Western-Light chemiluminescent detection system kit (Tropix, Bedford, Mass.) according to the manufacturer's instructions. Antibodies were used at 1/500 to 1/1,500 dilutions. The chemiluminescent signal was captured by exposure of blots to film.

For quantitative immunoblot analyses, 2.5-μl aliquots of cultures and five standards of PhaP (55, 27.5, 13.75, 6.88, and 3.44 ng) were included for each SDS-PAGE gel. A Hewlett-Packard ScanJet 4C desktop scanner and Deskscan 2.0 and Adobe Photoshop 5.0 software were used to convert signal on film to TIFF files. Automatic contrast adjustment was turned off during scanning. Quantitation of signal was performed on a Macintosh computer using the public domain NIH Image 1.60 program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RESULTS

Construction of *phaC* gene replacement strains. To test whether PhaP accumulation is dependent on PHB production in *R. eutropha* cells, we constructed a set of five *phaC* gene replacement plasmids and the corresponding *R. eutropha* gene replacement strains (Table 1). In the first strain, Re1017, the *phaC$_{Re}$* ORF has been precisely deleted. In the second and third strains, Re1031 and Re1036, the *phaC$_{Re}$* ORF has been replaced with the *phaEC$_{Cv}$* and *phaC$_{Cv}$* alleles, both of which encode active PHA synthase (20). In the fourth and fifth strains, Re1022 and Re1058, the *phaC$_{Re}$* ORF has been replaced with the *phaC$_{Re}$* C319A and *phaEC$_{Cv}$* C149A alleles, both of which encode inactive PHA synthase (9, 20). These strains were generated to determine how specific changes in the PHA synthase genes affect PhaP accumulation. The *phaC* precise-deletion strain was generated to test whether a nonpolar null mutation of *phaC* would be sufficient to block PhaP accumulation. The *phaEC$_{Cv}$* and *phaC$_{Cv}$* strains were generated to test whether expression of heterologous, active PHA synthases is sufficient to promote PhaP accumulation. Finally, the *phaC$_{Re}$* C319A and *phaEC$_{Cv}$* C149A strains were generated to establish whether expression of inactive PHA synthases could promote PhaP accumulation.

TABLE 3. Analysis of PHB production in *R. eutropha* wt and *phaC* gene replacement strains[a]

Strain	Culture OD_{600}	cdw (mg/ml of culture)	PHB (mg/ml of culture)	PHB (% cdw)
wt	10.2 ± 0.79	1.82 ± 0.15	1.45 ± 0.12	80
phaC precise deletion	0.94 ± 0.28	0.29 ± 0.01	<0.0003	<0.1[b]
phaEC$_{Cv}$	11.0 ± 0.21	1.84 ± 0.01	1.67 ± 0.02	91
phaC$_{Cv}$	1.36 ± 0.04	0.38 ± 0.11	0.0065 ± 0.0026	1.7
phaC$_{Re}$ C319A	1.10 ± 0.06	0.22 ± 0.06	<0.0003	<0.1
phaEC$_{Cv}$ C149A	1.17 ± 0.07	0.41 ± 0.01	<0.0003	<0.1

[a] Data correspond to analyses of strains inoculated in PHB production medium at an initial OD_{600} of 1.0 and cultivated for 72 h. Data for wt represent six independent cultures. Data for all other strains represent two independent cultures. The limit of detection is 0.0003 mg of PHB/ml of culture.
[b] Lower limit of detection for PHB = 0.1% cdw.

PHB production requires active PHA synthases. As the first step toward testing whether PhaP accumulation depends on PHB production in *R. eutropha*, we determined the amount of PHB produced by each strain. For these analyses, strains were cultivated in PHB production medium and were harvested after 72 h of cultivation, and the PHB present in cells was quantitated by the sulfuric acid/high-pressure liquid chromatography method (13). The wt strain was analyzed in parallel for comparison. Results for culture OD_{600}, cell dry weight (cdw), and PHB quantitation are shown in Table 3. The results indicate that the strains expressing active PHA synthase produce detectable amounts of PHB, whereas those expressing inactive or no PHA synthase produce no detectable PHB. The observation that the *phaEC$_{Cv}$* strain produces high amounts of PHB (91% cdw) like the wt strain (80% cdw) indicates that the heterologous PhaEC$_{Cv}$ PHA synthase can functionally replace PhaC$_{Re}$ in *R. eutropha*. The observation that the *phaC$_{Cv}$* strain produces much less PHB (1.7% cdw) is consistent with the report of Müh et al. (20) that PhaC$_{Cv}$ in vitro exhibits 1/150 the activity of PhaEC$_{Cv}$. Thus, the PhaE$_{Cv}$ cosynthase is also required for production of PHB to high levels by the *C. vinosum* PHA synthase during expression in *R. eutropha*.

Interestingly, the results also indicate that strains that lack highly active PHA synthase exhibit little or no growth during cultivation under conditions that promote the production of

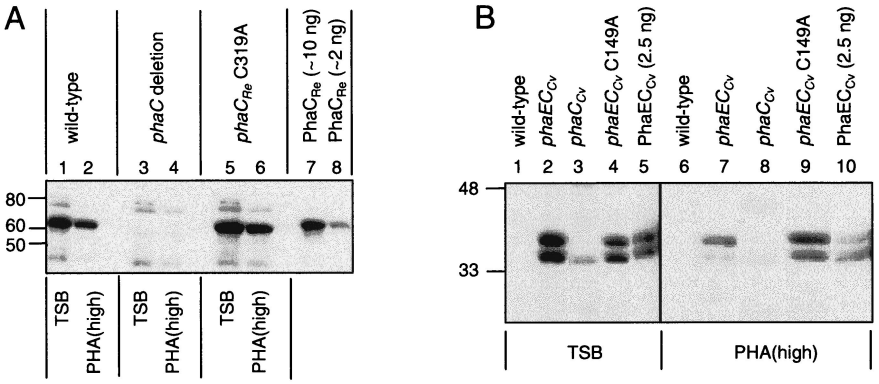

FIG. 1. (A) Accumulation of $PhaC_{Re}$ in *R. eutropha* wt, *phaC* deletion, and $phaC_{Re}$ C319A strains, as detected by anti-$PhaC_{Re}$ antibody. Proteins were separated by SDS–10% PAGE and were subjected to immunoblot analysis. Molecular-mass standards are indicated in kilodaltons. Cells from *R. eutropha* cultures were harvested after cultivation for 48 h in TSB or PHA(high). Bacterial samples correspond to cells from 10 μl of culture diluted to an OD_{600} of 1.0. Purified $PhaC_{Re}$ was included as a positive control. (B) Accumulation of $PhaEC_{Cv}$ in *R. eutropha* wt, $phaEC_{Cv}$, $phaC_{Cv}$, and $phaEC_{Cv}$ C149A strains, as detected by anti-$PhaEC_{Cv}$ antibody. Samples were analyzed as described above. Purified $PhaEC_{Cv}$ was included as a positive control. Note that the data correspond to two blots (first blot, lanes 1 to 5; second blot, lanes 6 to 10).

PHB to high levels (Table 3; see OD_{600} and cdw). Measurements of CFU in cultures in these and additional experiments indicate that the *phaC* deletion, $phaC_{Re}$ C319A, $phaEC_{Cv}$ C149A, and $phaC_{Cv}$ strains typically remain viable over the course of these types of cultivations (data not shown) but exhibit little or no increase in biomass. This observation raises the possibility that defects in PHB production might affect PhaP accumulation indirectly, by negatively affecting cell growth.

Active and inactive PHA synthases accumulate in the *R. eutropha* strains. As the second step toward testing whether PhaP accumulation depends on PHB production in *R. eutropha* cultures, the extent to which PHA synthases accumulate in each of the strains was determined by immunoblotting. We were interested in determining the stability of inactive PHA synthases under different growth conditions. We focused on two growth media, TSB and PHA(high), in which the wt strain accumulates PHB to low and high levels, respectively (37). Cells were harvested after 48 h of cultivation, and the presence of PHA synthase was detected by immunoblot analyses with anti-$PhaC_{Re}$ and anti-$PhaEC_{Cv}$ antibodies. The wt strain was analyzed in parallel for comparison. The results are shown in Fig. 1.

PHA synthases are detectable in strains cultivated in TSB or PHA(high), regardless of their activity. The anti-$PhaC_{Re}$ antibody detects a 64-kDa protein consistent with $PhaC_{Re}$ in the wt (Fig. 1A, lanes 1 and 2) and $phaC_{Re}$ C319A strains (Fig. 1A, lanes 5 and 6) but not in the *phaC* deletion strain (Fig. 1A, lanes 3 and 4). The anti-$PhaEC_{Cv}$ antibody detects a 39-kDa protein, consistent with $PhaC_{Cv}$, that is present in the $phaEC_{Cv}$, $phaC_{Cv}$, and $phaEC_{Cv}$ C149A strains (Fig. 1B, lanes 2 to 4, 7, and 9). The detection of the 39-kDa protein in the $phaC_{Cv}$ strain cultivated in PHA(high) requires prolonged exposure of immunoblots to film (data not shown). The anti-$PhaEC_{Cv}$ antibody also detects a 41-kDa protein, consistent with $PhaE_{Cv}$, that is present in the $phaEC_{Cv}$ and $phaEC_{Cv}$ C149A strains (Fig. 1B, lanes 2, 4, 7, and 9) but not in the $phaC_{Cv}$ strain (Fig. 1B, lanes 3 and 8). The results indicate some variability in amounts of the different synthases in *R. eutropha*. The basis for this variability has not been determined. Importantly, $PhaC_{Re}$ C319A and $PhaEC_{Cv}$ C149A can both be expressed detectably in *R. eutropha*; thus, the corresponding gene replacement strains can be used to test whether the physical presence of PHA synthase might be sufficient to enable PhaP accumulation.

PhaP accumulation is dependent on expression of active PHA synthase. We proceeded to test whether expression of heterologous and/or inactive synthases is sufficient to promote PhaP accumulation. We also tested whether PhaP accumulation is altered in particular strains due to altered cell growth or an inability to produce PHB. Strains were cultivated in TSB, PHA(med), and PHA(high), and cultures were analyzed after 48 h. In parallel, the wt strain and the *phaC3*::Tn*5* and *phaP* deletion strains were analyzed as positive and negative controls, respectively. Results for culture OD_{600} and immunoblot analyses are shown in Fig. 2.

The results indicate that PhaP accumulation is dependent on expression of active PHA synthase. Anti-PhaP antibody detects a 24-kDa protein, consistent with PhaP, in the wt strain (Fig. 2A, lanes 1 to 3), the $phaEC_{Cv}$ strain (Fig. 2A, lanes 16 to 18), and, to a lesser extent, in the $phaC_{Cv}$ strain (Fig. 2A, lane 21). This 24-kDa protein is not detected in the *phaC* deletion, $phaC_{Re}$ C319A, and $phaEC_{Cv}$ C149A strains nor in the *phaC3*::Tn*5* and *phaP* deletion strains (Fig. 2A, lanes 4 to 15 and 22 to 24). The observation that PhaP accumulates in the $phaEC_{Cv}$ and the $phaC_{Cv}$ strains suggests that expression of any active PHA synthase is sufficient to enable PhaP accumulation. The observation that PhaP accumulates to low levels or fails to accumulate in the $phaEC_{Cv}$ and the $phaC_{Cv}$ strains during cultivation in TSB is consistent with our previous report that PhaP accumulates only transiently in the wt strain in TSB (37). The observation that PhaP is not detectable in strains that are genetically blocked for PHB synthesis, independent of the precise nature of the genetic block, suggests that PhaP accumulation is strictly dependent on the ability of cells to express active PHA synthase. The observation that PhaP fails to accumulate in strains that are genetically blocked in PHB synthesis, even when the strains are cultivated in PHA(med) and thus are exhibiting substantial growth (Fig. 2B), argues against the possibility that *phaC* mutations block PhaP accumulation indi-

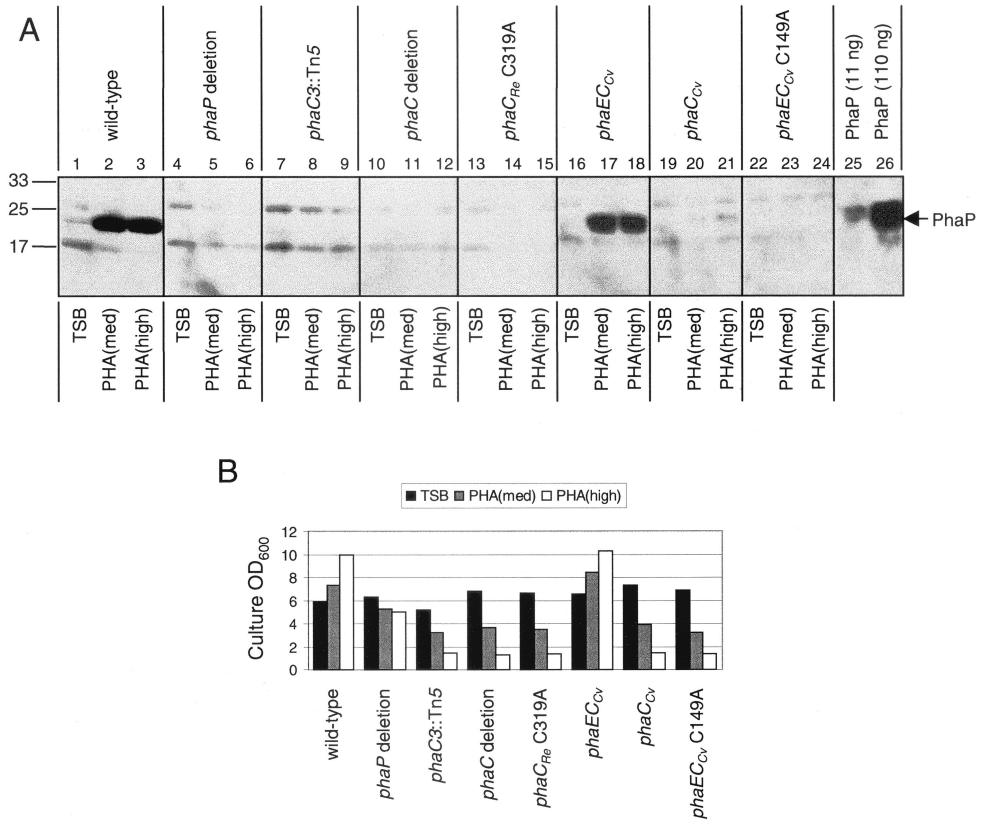

FIG. 2. (A) Accumulation of PhaP in *R. eutropha* wt, *phaP* deletion, *phaC3*::Tn*5*, *phaC* deletion, $phaC_{Re}$ C319A, $phaEC_{Cv}$, $phaC_{Cv}$, and $phaEC_{Cv}$ C149A strains. Proteins were separated by SDS–15% PAGE and were subjected to immunoblot analysis for detection of PhaP. Molecular-mass standards are indicated in kilodaltons. Cells from *R. eutropha* cultures were harvested after cultivation for 48 h in TSB, PHA(med), and PHA(high). Bacterial samples correspond to cells from 10 μl of culture diluted to an OD_{600} of 1.0. Data correspond to three blots (first blot, lanes 1 to 3; second blot, lanes 4 to 15; third blot, lanes 16 to 26). Purified PhaP was included as a positive control on each blot. Blots were exposed to film for 5, 10, and 30 min. Data correspond to 10-min exposures for the first and second blots and a 30-min exposure for the third blot. (B) Measurements of OD_{600} for *R. eutropha* strains after cultivation for 48 h in TSB, PHA(med), and PHA(high). Data were extrapolated from 10-fold dilutions of cultures.

rectly due to effects on growth. Taken together, these observations strongly suggest that PhaP accumulation in a given strain is specifically dependent on PHB production in that strain.

PhaP accumulation is regulated at the level of PhaP synthesis. Our studies are consistent with the possibilities that PhaP accumulation is regulated at the level of PhaP synthesis, PhaP degradation, or both. To test for regulation of PhaP accumulation at the level of PhaP synthesis, we constructed a *phaP-gfp* translational fusion and tested the effects of growth conditions and mutations in *phaC* on expression of this fusion in *R. eutropha*. We reasoned that a *phaP-gfp* translational fusion could serve as a useful reporter for PhaP synthesis, based on the assumption that GFP would not be subjected to degradation by any mechanisms which may exist to specifically degrade PhaP. We designed the *phaP-gfp* fusion such that it includes transcriptional and translational start signals of *phaP* and thus corresponds to a translational fusion (29) and such that it encodes a variant of GFPmut2 (N-Met-Val-Glu-GFPmut2-C) in place of PhaP. *R. eutropha* strains in which the *phaP* gene has been replaced by the *phaP-gfp* translational fusion were constructed in the wt and *phaC3*::Tn*5* backgrounds to yield a *phaP-gfp* strain, designated Re1001, and a *phaP-gfp phaC3*::Tn*5* strain, designated Re1007.

To test regulation of expression of the *phaP-gfp* fusion, the *phaP-gfp* and *phaP-gfp phaC3*::Tn*5* strains were cultivated in TSB, PHA(med), and PHA(high); cells were harvested after 48 h of cultivation; and the presence of GFP in cells was detected by immunoblot analysis with anti-GFP antibodies. An *E. coli* strain carrying the *phaP-gfp* fusion on a plasmid and a strain carrying the corresponding vector without the *phaP-gfp* fusion were included as positive and negative controls, respectively. The *R. eutropha* wt and *phaC3*::Tn*5* strains were also analyzed in parallel as negative controls. Results for culture OD_{600} and immunoblot analyses are shown in Fig. 3. These results parallel those of PhaP immunoblot analyses (Fig. 2). Specifically, the anti-GFP antibody recognizes a 27-kDa protein, consistent with GFP, that accumulates to much higher levels in the *phaP-gfp* strain (Fig. 3A, lanes 7 to 9) than in the *phaP-gfp phaC3*::Tn*5* strain (Fig. 3A, lanes 10 to 12). Unfortunately, the anti-GFP antibodies cross-react with a 27-kDa *R. eutropha* protein (Fig. 3A, lanes 1 to 6), obscuring a lower limit of detection for GFP. Nonetheless, the results indicate that PhaP accumulation is regulated at least in part at the level

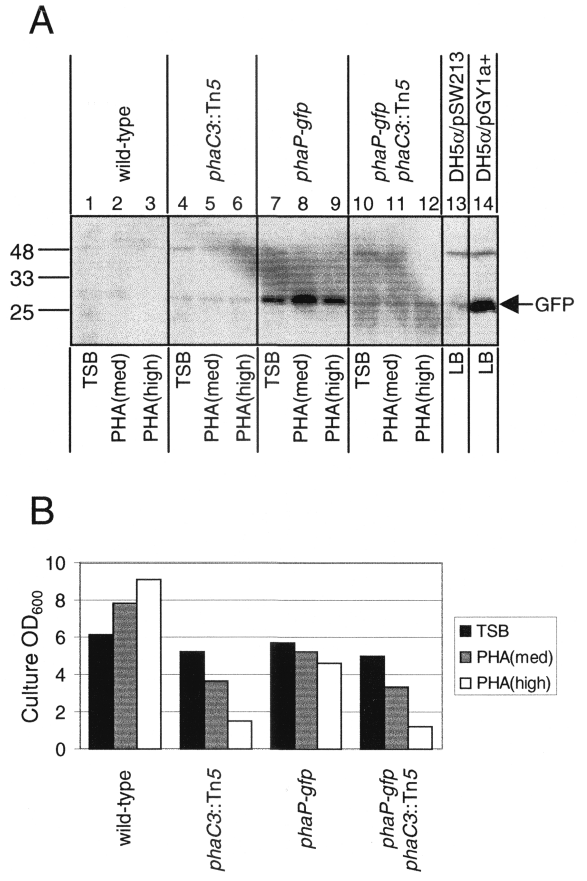

FIG. 3. (A) Accumulation of GFP in wt, *phaC3*::Tn*5*, *phaP-gfp*, and *phaP-gfp phaC3*::Tn*5* *R. eutropha* strains. Proteins were separated by SDS–15% PAGE and were subjected to immunoblot analysis for detection of GFP. Molecular-mass standards are indicated in kilodaltons. Cells from *R. eutropha* cultures were harvested after cultivation for 48 h in TSB, PHA(med), and PHA(high). Bacterial samples correspond to cells from 10 μl of culture diluted to an OD_{600} of 1.0. The *E. coli* strains DH5α/pGY1a+, which carries the *phaP-gfp* fusion on a plasmid, and DH5α/pSW213, which carries the corresponding vector lacking the *phaP-gfp* fusion, were included as positive and negative controls, respectively. (B) Measurements of OD_{600} for *R. eutropha* strains after cultivation for 48 h in TSB, PHA(med), and PHA(high). Data were extrapolated from 10-fold dilutions of cultures.

of PhaP synthesis. In addition, the observation that the *phaP-gfp phaC3*::Tn*5* strain exhibits substantial increases in OD_{600} during cultivation in TSB and PHA(med) (Fig. 3B) suggests that the strain fails to express the *phaP-gfp* fusion not due to lack of growth but rather due to lack of PHB production.

We recently became aware of a study, reported by Steinbüchel's group in a conference paper (33), which supports regulation at the level of PhaP synthesis. This study indicates that expression of the *phaP* gene is regulated at the level of transcription in a manner dependent on PHB production in cells, based on S1 nuclease analyses. Our results are consistent with those reported in this study, except in one important respect. The observation of Steinbüchel's group that the wt strain produces little or no *phaP* transcript during cultivation in rich medium (33), along with their earlier report that the wt strain produces no PHB and no PhaP protein during cultivation in rich medium (35), contradicts recent observations of our group and others that the wt strain produces both PHB (27, 37) and PhaP (37) during cultivation in rich medium.

PhaP stability is dependent on the presence of PHB in cells. The observation that PhaP accumulation is regulated at the level of PhaP synthesis does not rule out the possibility that PhaP accumulation may also be regulated at the level of PhaP degradation. In fact, we recently reported that PhaP levels rise and fall with PHB levels in the wt strain cultivated in TSB (37), which suggests that PhaP accumulation is also regulated at the level of PhaP degradation. The basis for this degradation is not known. One possibility is that net PHB utilization is sufficient to trigger PhaP degradation. Alternatively, intracellular PHB may need to decrease below some minimal threshold level in order to trigger PhaP degradation.

To distinguish between these possibilities, the cells of the wt strain were first cultivated in PHA(med) or PHA(high) for 72 h to allow the accumulation of PHB and PhaP and were then washed, diluted fourfold, and cultivated in PHA(no carbon) for an additional 72 h to trigger partial utilization of intracellular PHB. PHB and PhaP were quantitated over time. The results are shown in Fig. 4. PhaP levels remain constant for cells that were previously cultivated in PHA(med) (Fig. 4A) and, somewhat surprisingly, increase for cells that were previously cultivated in PHA(high) (Fig. 4B). This is the case even though PHB levels decrease during these cultivations (Fig. 4): PHA(med), 51 to 21% cdw; PHA(high), 92 to 48% cdw. Taken together with results from our previous analyses of the wt strain cultivated in TSB (37), these results tend to rule out the possibility that net PHB utilization is sufficient to trigger PhaP degradation. Instead, the results suggest that net PhaP degradation begins only after levels of PHB have decreased below a certain minimal level in cells (PHB level $< \sim$20% cdw, for example). Furthermore, these results indicate that net PhaP synthesis can occur in cells simultaneously with net PHB utilization. This observation contradicts models whereby PhaP accumulation is strictly dependent on PHB synthesis and suggests rather that the presence of a certain minimal amount of PHB in cells (PHB level $> \sim$50% cdw, for example) is sufficient to trigger PhaP synthesis.

PhaP accumulation is regulated at the level of individual cells. Our studies thus far do not distinguish between the possibilities that PhaP accumulation is regulated at the level of individual cells or populations of cells. If PhaP accumulation is regulated by a mechanism involving direct detection of PHB, then PhaP accumulation in a given cell will strictly depend on the production of PHB in that cell. In contrast, if PhaP accumulation is regulated by a mechanism involving indirect detection of PHB—for example, based on the presence of particular metabolites or extracellular signals in culture supernatants—then PhaP accumulation in a given cell may depend only on the production of PHB in a subset of cells in the surrounding population. We were particularly interested in distinguishing between these two possibilities, given the suggestion of Campos-García et al. (2) that in *Pseudomonas aeruginosa* the expression of a ketoacyl reductase implicated in PHA synthesis may be regulated by quorum sensing and given that the potential usefulness of PhaP expression as a marker for PHB production in cells would depend on knowing the basis for regulation of PhaP accumulation.

To distinguish between regulation of PhaP accumulation at

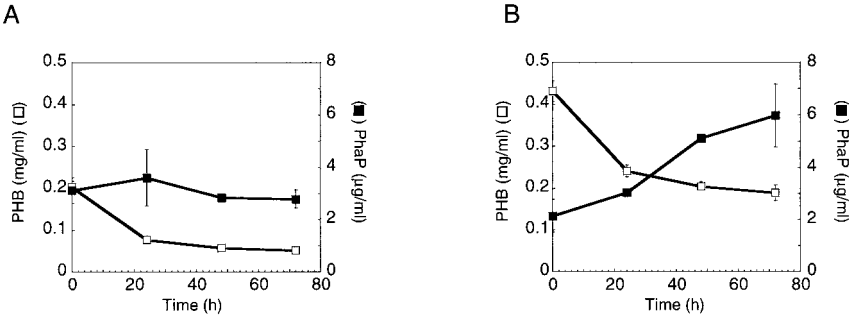

FIG. 4. Comparison of levels of PhaP versus PHB for wt strain cultivated under PHB utilization conditions (PHA[no carbon]) for 72 h. Cells were cultivated in PHA(med) (A) or PHA(high) (B) (200 ml) for 72 h, washed, diluted fourfold, and were then cultivated in PHA(no carbon) for 72 h. Time zero corresponds to start of cultivation in PHA(no carbon). All data points represent average value for two cultures (error bars represent standard deviations).

the level of individual cells versus populations of cells, we tested whether the presence of cells of the *phaP* deletion strain, which produce PHB (37), could cause the accumulation of PhaP in cells of the *phaC* deletion strain, which normally do not express PhaP. We reasoned that if PhaP accumulation is regulated at the level of individual cells, then PHB production by the *phaP* deletion strain would not be sufficient to cause PhaP accumulation in the *phaC* deletion strain. In contrast, if PhaP accumulation is regulated at the level of populations of cells, then PHB production by the *phaP* deletion strain would be sufficient to cause PhaP accumulation in the *phaC* deletion strain. For this experiment, equal amounts of the *phaP* and *phaC* deletion strains (based on culture OD_{600}) were combined, cultivated together in PHA(med) for 48 h, and subjected to PhaP immunoblot analysis. PHA(med) was used as the growth medium because it is sufficient to trigger the production of substantial amounts of PHB by the *phaP* deletion strain (37) and to promote substantial growth of the *phaC* deletion strain (Fig. 3). Cocultivation of the wt strain with the *phaP* or *phaC* deletion strain and cultivation of each strain alone were conducted in parallel for comparison. Results for the immunoblot analyses are shown in Fig. 5.

No signal consistent with PhaP is observed for cocultivation of the *phaP* deletion strain and *phaC* deletion strains (Fig. 5, lane 1), indicating that the presence of cells that can produce PHB in a culture is not sufficient to trigger PhaP accumulation in other cells in the same culture. The observation that PhaP accumulates to detectable levels in cultures of the wt strain cultivated with either the *phaP* deletion strain (Fig. 5, lane 3) or the *phaC* deletion strain (Fig. 5, lane 2) tends to rule out the possibility that the presence of the *phaP* or *phaC* deletion strains interferes with PhaP expression or PHB production in other cells in the same cultures. The same results were observed in an independent experiment in which coinoculations of cells of the *phaP* and *phaC* deletion strains were conducted in ratios ranging from 1:9 to 9:1 (data not shown). The results suggest that accumulation of PhaP in a given cell depends on the production and/or accumulation of PHB in that cell.

DISCUSSION

Based on our results we propose a model for regulation of PhaP phasin accumulation in *R. eutropha*. According to our model, net PhaP synthesis is triggered by either of two conditions, net synthesis of PHB or the presence of relatively high amounts of intracellular PHB (>50% cdw). Our results suggest that *R. eutropha* has evolved a regulatory mechanism that can detect either of these conditions. Net PhaP degradation is triggered by the combination of two conditions, net utilization of PHB and the presence of relatively low amounts of PHB in cells. Our results are consistent with the possibilities that the combination of these conditions triggers expression of a mechanism for degradation of PhaP in cells or that such a mechanism is expressed constitutively but that PhaP is susceptible to degradation only when the combination of conditions applies.

How might PhaP synthesis be regulated? One possibility is that cells express a negative regulator of phasin expression and that this negative regulator is titrated by binding to intracellular PHB. Prior to PHB synthesis, the negative regulator would prevent phasin expression. During net synthesis of PHB, the negative regulator would bind the newly synthesized PHB, resulting in derepression of phasin expression. During net utilization of PHB, the negative regulator would remain bound to the PHB until the PHB had decreased below a certain level (~50% cdw). At this point the negative regulator would begin to be released and would again repress phasin expression. Such a model could explain how net PHB synthesis or the presence

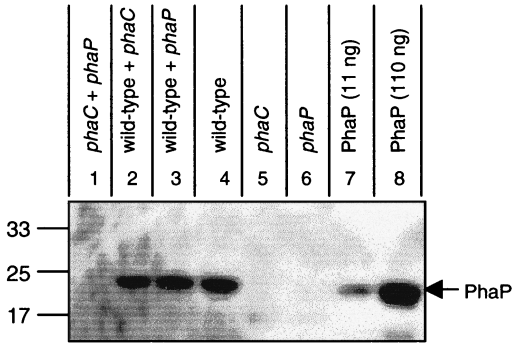

FIG. 5. Accumulation of PhaP in cultures of wt, *phaC* deletion, and *phaP* deletion strains, cultivated alone or cocultivated in pairs. Proteins were separated by SDS–15% PAGE and were subjected to immunoblot analysis for detection of PhaP. Molecular-mass standards are indicated in kilodaltons. Cells from *R. eutropha* cultures were harvested after cultivation for 48 h in PHA(med). Bacterial samples correspond to cells from 10 μl of culture diluted to an OD_{600} of 1.0. Purified PhaP was included as a positive control.

of large amounts of PHB in cells could trigger PhaP accumulation.

This model for negative regulation of phasin accumulation in *R. eutropha* seems particularly attractive, given that a regulatory system of this type has been proposed previously by Prieto et al. (24) for PhaF-mediated regulation of PHA synthase expression in *P. oleovorans* and has been anticipated by Maehara et al. (17) for PhaR-mediated regulation of phasin accumulation in *P. denitrificans*. Genetic evidence suggests that PhaF and PhaR may be transcriptional repressors that are titrated from DNA by intracellular PHA (17, 24). Given the observation of Maehara et al. (17) that homologs of PhaR occur in many PHA-producing strains, including *R. eutropha*, it seems likely that this type of regulatory mechanism will prove to be a general feature of PHA synthesis.

How might PhaP degradation be regulated? One possibility is that PhaP is protected from proteolytic degradation as long as it is bound to PHB granules and that once PHB decreases below a certain level (~20% cdw), PhaP protein is released into the cytosol and is degraded. This idea seems particularly attractive, given the report of Wieczorek et al. (35) that PhaP in *R. eutropha* cells is detected only associated with PHB granules and not in the cytosol.

Regulation of phasin accumulation is important for PHA production. Improved understanding of this regulation may be useful in efforts aimed at precisely manipulating the timing and levels of phasin expression in PHA-producing strains, which in turn should be useful in determining the precise nature of the role of phasins. The observation that phasin accumulation is regulated at the level of individual cells is particularly important because it suggests that a cell expresses phasin not as a general response to growth conditions or even to PHA production within other cells in the same population but strictly as a response to the presence of PHA within the given cell. Thus, expression of PhaP or a surrogate marker such as the *phaP-gfp* translational fusion could serve as an indicator of production of PHB, not just in cultures but in individual cells in a mixed population. This point could prove useful in efforts to optimize PHA synthases through genetic engineering.

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