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The *Ralstonia eutropha* PhaR Protein Couples Synthesis of the PhaP Phasin to the Presence of Polyhydroxybutyrate in Cells and Promotes Polyhydroxybutyrate Production

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Polyhydroxyalkanoates (PHAs) are polyoxoesters that are produced by many bacteria and that accumulate as intracellular granules. Phasins (PhaP) are proteins that accumulate during PHA synthesis, bind PHA granules, and promote further PHA synthesis. Interestingly, PhaP accumulation seems to be strictly dependent on PHA synthesis, which is catalyzed by the PhaC PHA synthase. Here we have tested the effect of the *Ralstonia eutropha* PhaR protein on the regulation of PhaP accumulation. *R. eutropha* strains with *phaR*, *phaC*, and/or *phaP* deletions were constructed, and PhaP accumulation was measured by immunoblotting. The wild-type strain accumulated PhaP in a manner dependent on PHA production, and the *phaC* deletion strain accumulated no PhaP, as expected. In contrast, both the *phaR* and the *phaR phaC* deletion strains accumulated PhaP to higher levels than did the wild type. This result implies that PhaR is a negative regulator of PhaP accumulation and that PhaR specifically prevents PhaP from accumulating in cells that are not producing PHA. Transfer of the *R. eutropha phaR*, *phaP*, and PHA biosynthesis (*phaCAB*) genes into a heterologous system, *Escherichia coli*, was sufficient to reconstitute the PhaR/PhaP regulatory system, implying that PhaR both regulates PhaP accumulation and responds to PHA directly. Deletion of *phaR* caused a decrease in PHA yields, and a *phaR phaP* deletion strain exhibited a more severe PHA defect than a *phaP* deletion strain, implying that PhaR promotes PHA production and does this at least partially through a PhaP-independent pathway. Models for regulatory roles of PhaR in regulating PhaP and promoting PHA production are presented.

Polyhydroxyalkanoates (PHAs) are polyoxoesters that are produced by a wide range of bacteria as carbon storage compounds and that accumulate intracellularly as amorphous granules (2, 11, 21). Three classes of proteins, PHA synthase (PhaC), PHA depolymerase (PhaZ), and phasin (PhaP), play important roles in PHA metabolism. PHA synthases catalyze the conversion of *R*-3-hydroxyalkanoyl coenzyme A molecules to PHAs (4, 11, 16). PHA depolymerases hydrolyze PHAs to yield oligomers and/or monomers of hydroxyalkanoates (5, 7, 22). Phasins accumulate during PHA synthesis, bind PHA granules, and promote further PHA synthesis in a manner that is poorly understood (6, 13, 15, 17, 25, 27). Despite similarities in regulation and function, phasins from different bacterial species are not homologs. This study focuses on the mechanism that couples phasin synthesis with the presence of PHA in cells.

Several recent studies have shed light on phasin regulation. Maehara et al. (13) have identified a *Paracoccus denitrificans* protein, designated PhaR, that is an excellent candidate for a negative regulator that couples PhaP phasin synthesis to PHA production by preventing PhaP accumulation in the absence of PHA. The *P. denitrificans phaR* gene was identified based on its proximity to the *phaP* and *phaC* genes (13). In a heterologous system, *Escherichia coli*, PhaP of *P. denitrificans* accumulates to lower levels in the presence of the *phaR* gene (13), and PhaR binds the *phaP* promoter region and blocks *phaP* expression in vitro (12). One simple model for PhaR function is that PhaR contains a domain that binds DNA and a second domain that binds PHA or a factor associated with PHA. In the absence of PHA, PhaR binds to and represses transcription of *phaP*. In the presence of PHA, PhaR is titrated from *phaP* and thus enables transcription of *phaP*.

Many PHA-producing bacteria encode a PhaR homolog (13), suggesting that PhaR may be important for phasin regulation and PHA biosynthesis. *Ralstonia eutropha* is an excellent system to study phasin regulation because it is amenable to standard gene replacement techniques (20, 24), it is the only strain for which phasin mutants have been generated (25, 27), and it produces polyhydroxybutyrate (PHB) under a range of cultivation conditions. In *R. eutropha* the *phaR* gene (originally designated ORF1 [24]) is located downstream of the PHA biosynthetic operon *phaCAB* and is not linked to *phaP*. Here we report that *R. eutropha* PhaR is a negative regulator of PhaP accumulation and that PhaR prevents PhaP accumulation in the absence of PHB.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. The strains and plasmids used in this study are listed in Table 1. The oligonucleotides are as follows: dGCGATTTC GCCCGACGCACCCAGCACTAA, H508E; dAGCTTGGATCCGATGCGA GCGCTGCATACC, phaC2; dAGCTTGGATCCGGCGCTCATGTTTTCCT GG, phaC5; dCCGAGGATCCATCGCCGGACAAGGCAGC, phaP2; dCTAG CGAATTCGGATCCGCAATCGCGCATCGTTG, phaP4; dGCCGAGGATC CTTTCGCGGGCCGTCAAGGC, phaR1; dCCCGCCGCTGCCAGTGTCCG

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

Strain or plasmid	Description[b]	Reference or source
Strains		
R. eutropha		
Ae H16	wt, Gm^r, also termed DSM 428 and ATCC 17699	ATCC 17699
Re1001	*phaP-gfp* translational fusion gene replacement strain	26
Re1034	Δ*phaC* strain	26
Re1052	Δ*phaP* strain	27
Re1099	Δ*phaR* strain, derived from Ae H16/pGY95	This study
Re1101	Δ*phaC* Δ*phaR* strain, derived from Re1034/pGY95	This study
Re1102	Δ*phaP* Δ*phaR* strain, derived from Re1052/pGY95	This study
Re1103	Δ*phaR phaP-gfp* translational fusion strain, derived from Re1001/pGY95	This study
Re1105	Δ*phaC phaP-gfp* translational fusion strain, derived from Re1001/pGY46	This study
Re1106	Δ*phaC* Δ*phaR phaP-gfp* translational fusion strain, derived from Re1103/pGY46	This study
E. coli		
DH5α	Strain for ligation, cloning, and heterologous expression of PHA genes	New England Biolabs
Plasmids		
pAeT41	5-kb *Sma*I/*Eco*RI *phaCAB* DNA cloned into *Sma*I/*Eco*RI sites of pUC18	16
pCR2.1-TOPO	High-copy-number plasmid used for cloning, confers Ap^r and Km^r	Invitrogen
pGY1a+	*phaP-gfp* translational fusion cloned into pSW213, positive control for GFP expression	26
pGY4+	1.5-kb *phaP* DNA in *BamH*I site of pSW213, *phaP* in same orientation as *lac* promoter	This study
pGY4−	1.5-kb *phaP* DNA in *BamH*I site of pSW213, *phaP* in opposite orientation as *lac* promoter	This study
pGY46	Δ*phaC* gene replacement plasmid, confers Km^r	26
pGY95	Δ*phaR* gene replacement plasmid, confers Km^r	This study
pGY104−	1.35-kb *phaR* DNA in *Bgl*II site of pSW213, *phaR* in opposite orientation as *lac* promoter	This study
pGY105	1.5-kb *phaP* DNA in *Bam*HI site of pGY104−, *phaP* oriented divergently from *phaR*	This study
pJQ200mp18Km	Derivative of pJQ200mp 18, Gm^r gene disrupted, confers Km^r	27
pSW213	Low-copy-number plasmid, confers Tc^r	3
pUC18	High-copy-number plasmid used for cloning, confers Ap^r	Invitrogen

[a] Plasmids constructed in this study that were used only as intermediates for construction of other plasmids are described only in the Materials and Methods section.
[b] Abbreviations: Ap^r, Gm^r, Km^r, and Tc^r, ampicillin, gentamicin, kanamycin, and tetracycline resistance, respectively.

GTGCTCTGTCCTTGTGTC, phaR2; dGACACAAGGACAGAGCACCGGA CACTGGCAGCGGCGGG, phaR3; dCCGGAGGATCCATGAAGGAACC AACCCGC, phaR4; dGCGCAACCATATGGCCACGACCAAAAAAGGC, phaR5; dCCGGAGGATCCAGTGTCTTACTTCTTGTCCG, phaR6; and dGC CTTGACCGAGCTGGCCGAT, Seq376. Engineered restriction sites are underlined. H508E and phaP2 include mismatches relative to the correct sequences of *phaC* and *phaP*, respectively.

Growth media and antibiotics. *R. eutropha* strains were cultivated in Luria-Bertani (LB) medium (14), Tryptic Soy Broth-Dextrose Free (TSB) medium (Becton Dickinson Microbiology Systems, Cockeysville, Md.), PHA(med), and PHA(high). PHA(med) and PHA(high) are based on a minimal medium (16) supplemented with fructose (0.5 or 1%, respectively) and ammonium chloride (0.1 or 0.01%, respectively). *E. coli* strains were cultivated in LB medium. Antibiotics were added to growth media to the following final concentrations: for *R. eutropha*, gentamicin (10 μg/ml) and kanamycin (270 μg/ml); and for *E. coli*, ampicillin (100 μg/ml), gentamicin (10 μg/ml), kanamycin (25 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 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 *R. eutropha* immunoblot analyses, strains were cultivated in 2 ml of TSB in test tubes to saturation (24 to 30 h). Aliquots (100 μl) were transferred to 5 ml of TSB in test tubes and were cultivated to saturation (~16 h). Aliquots of washed cells were transferred to 5 ml of TSB, PHA(med), or PHA(high) to yield cultures with an initial optical density at 600 nm (OD_{600}) of 1.0 (~10^9 CFU/ml) and cultivated for 48 h. For *R. eutropha* PHB production and PhaP quantitation analyses, strains were cultivated as for immunoblot analyses but on a larger scale [growth in 4 ml of TSB in test tubes (36 h), then in 100 ml of TSB in 500-ml flasks (24 h), and finally in 200 ml of LB medium with 2% fructose or PHA(high) in 1-liter baffled flasks (72 h)]. For *E. coli* immunoblot analyses, strains were cultivated in 2.5 ml of LB(Ap Tc) (that is, LB medium containing ampicillin and tetracycline) in test tubes to saturation (24 h). Aliquots of washed cells were transferred to 5 ml of LB medium plus 2% glucose(Ap Tc) to yield cultures with an initial OD_{600} of 0.9 and cultivated 24 h. For *E. coli* PHB production analyses, strains were cultivated as for immunoblot analyses [growth in 5 ml of LB(Ap Tc) in test tubes (16 to 22 h) and then in 100 ml of LB medium plus 2% glucose(Ap Tc) in 500-ml flasks at initial OD_{600} of 0.15 (72 to 105 h)].

DNA preparation and manipulation. Standard approaches were used for preparation and manipulation of DNA, for transformation of *E. coli*, and for the PCR (1). Genomic DNA was prepared from *R. eutropha* strains as described previously (26). All constructs containing PCR products were confirmed by sequencing at the MIT Biopolymer Lab.

Construction of *phaR* precise deletion gene replacement plasmid pGY95. The 347- and 349-bp fragments of *R. eutropha* DNA corresponding to the regions immediately upstream and downstream of the *phaR* open reading frame (ORF), respectively, were amplified by PCR with phaR1 and phaR2 or with phaR3 and phaR4. The two PCR products were combined and amplified with phaR1 and phaR4 to yield a 0.7-kb PCR ligation product spanning from upstream to downstream of the *phaR* ORF, but lacking the *phaR* ORF, flanked by *Bam*HI sites. The 0.7-kb *Bam*HI fragment of the PCR product was cloned into the *Bam*HI site of pJQ200mp18Km to yield pGY95.

Construction of *phaR* and *phaC* gene replacement strains. Gene replacement was accomplished by adaptation of standard protocols (20, 24). The combinations of starting strains and plasmids used for construction of each strain are indicated in Table 1. Construction of Δ*phaR* strains was confirmed by PCR using oligonucleotides phaR5 and phaR6, which amplify the *phaR* ORF (*phaR*$^+$, 0.6 kb; Δ*phaR*, no product). Construction of Δ*phaC* strains was confirmed by PCR with oligonucleotides phaC2 and phaC5 (*phaC*$^+$, 2.3 kb; Δ*phaC*, 0.8 kb) and Seq376 and H508E (*phaC*$^+$, 1.2 kb; Δ*phaC*, no product).

Plasmid encoding *phaP*. Plasmid pGY4+ was constructed as follows. A 1.5-kb fragment of *R. eutropha* DNA, spanning from 762 bp upstream to 141 bp downstream of the *phaP* ORF, was amplified by PCR with the oligonucleotides phaP4 and phaP2, such that *Bam*HI sites were introduced at both ends of the fragment. The corresponding 1.5-kb *Bam*HI fragment was cloned into the *Bam*HI site of the multicloning site of pSW213, in the same orientation as the *lac* promoter present in the plasmid, to yield pGY4+. Plasmid pGY4− contains the same 1.5-kb *Bam*HI fragment cloned into the *Bam*HI site of pSW213 in the opposite orientation.

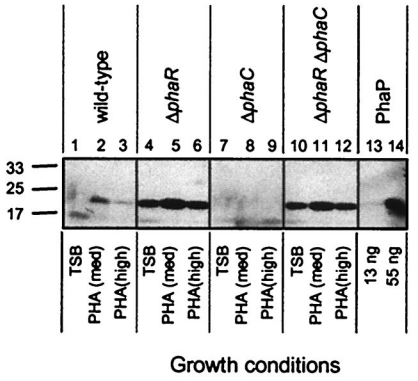

FIG. 1. Immunoblots of PhaP in *R. eutropha* strains. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were subjected to immunoblot analysis for detection of PhaP. Cells from *R. eutropha* cultures were harvested after cultivation for 48 h in TSB, PHA(med), or PHA(high). Bacterial samples correspond to cells from 10 μl of a culture diluted to an OD_{600} of 0.2. Purified PhaP was included as a control. Molecular mass standards are indicated in kilodaltons. Culture OD_{600} measurements were as follows: lane 1, 8.9; lane 2, 8.3; lane 3, 9.6; lane 4, 8.6; lane 5, 7.2; lane 6, 5.7; lane 7, 9.1; lane 8, 4.3; lane 9, 1.3; lane 10, 8.9; lane11, 3.8; lane 12, 1.8.

Plasmid encoding *phaR*. Plasmid pGY104− was constructed as follows. A 1.35-kb fragment of *R. eutropha* DNA, spanning from 347 bp upstream to 349 bp downstream of the *phaR* ORF, was amplified by PCR with the oligonucleotides phaR1 and phaR4, such that *Bam*HI sites were introduced at both ends of the fragment. This fragment was cloned into the *Afl*II site of pCR2.1-TOPO to yield the plasmid pB3. The 1.35-kb *Bam*HI fragment of pB3 was cloned into the *Bgl*II site in the multicloning site of pSW213, such that the *phaR* ORF is in the opposite orientation as the *lac* promoter on the plasmid, to yield pGY104−.

Plasmid encoding *phaP* and *phaR*. Plasmid pGY105 was constructed as follows. The 1.5-kb *Bam*HI fragment of pGY4+, containing *phaP*, was cloned into the *Bam*HI site of pGY104−, such that *phaP* is in the opposite orientation as *phaR* on the plasmid (gene order: *lac* promoter-*phaR* [reverse orientation]-*phaP*).

Quantitation of PHB in *R. eutropha* and *E. coli* cells. PHB was quantitated by the sulfuric acid–high-pressure liquid chromatography method of Karr et al. (8) with some modifications (26).

Recombinant PhaP, antibodies, and immunoblot analyses. Recombinant PhaP was purified as previously described (27) and quantitated based on its extinction coefficient (PhaP $\varepsilon_{280} = 5{,}687\ M^{-1}\ cm^{-1}$) as determined by measurements of mass (determined by amino acid analysis) and A_{280}. Preparation and purification of antibodies against PhaP, immunoblotting, and quantitation of PhaP in cells were conducted as described previously (26). Anti-green fluorescent protein (GFP) antibodies were obtained from Clontech (Palo Alto, Calif.).

RESULTS

PhaR negatively regulates PhaP accumulation. To determine whether *R. eutropha* PhaR regulates PhaP accumulation, a Δ*phaR* strain was constructed by precise deletion of the *phaR* ORF in the wild-type (wt) strain H16. An isogenic Δ*phaR* Δ*phaP* strain was also constructed by deletion of the *phaR* ORF in a Δ*phaP* strain. Strains were cultivated in TSB, PHA-(med), and PHA(high), which are growth media that promote the production of PHB to low, intermediate, or high levels, respectively (27). After 48 h, aliquots of culture, each normalized based on the OD_{600}, were subjected to immunoblot analyses for detection of PhaP. The wt and Δ*phaR* strains yielded equivalent culture OD_{600} readings for TSB and PHA(med) (Fig. 1), suggesting similar growth and/or PHA accumulation rates (10, 26). The Δ*phaR* strain, however, yielded much higher signal for the ~24-kDa PhaP protein than did the wt strain (Fig. 1, lanes 4 to 6 versus lanes 1 to 3). These observations suggest that PhaR negatively regulates PhaP accumulation across a range of growth conditions. The Δ*phaP* and Δ*phaR* Δ*phaP* strains yielded no signal for PhaP, as expected (data not shown).

PhaR couples PhaP accumulation to the production of PHB in individual cells. To test whether PhaR may prevent PhaP accumulation in the absence of PHB, a Δ*phaR* Δ*phaC* strain, which is incapable of producing PHB, was constructed, and PhaP accumulation was measured. The Δ*phaC* and Δ*phaR* Δ*phaC* strains exhibited comparable culture OD_{600} readings under each cultivation condition (Fig. 1). However, while the Δ*phaC* strain produced no detectable PhaP (Fig. 1, lanes 7 to 9), the Δ*phaR* Δ*phaC* strain produced large amounts of PhaP, equivalent to levels produced by the Δ*phaR* strain (Fig. 1, lanes 10 to 12). Deletion of *phaR* completely uncouples PhaP accumulation from PHB production in *R. eutropha*.

PhaR regulates PhaP accumulation at the level of PhaP synthesis. To test whether PhaR may regulate PhaP accumulation at the level of PhaP synthesis, Δ*phaR phaP-gfp*, Δ*phaC phaP-gfp*, and Δ*phaR* Δ*phaC phaP-gfp* strains were constructed, and GFP expression was measured. The *phaP-gfp* fusion is designed to reveal regulation of *phaP* at the level of transcription and/or translational initiation based on expression of GFP (26). Strains were cultivated in TSB and PHA(high) and analyzed for GFP expression by immunoblot. The Δ*phaR phaP-gfp* and Δ*phaR* Δ*phaC phaP-gfp* strains expressed the ~27-kDa GFP protein to approximately the same extent (Fig. 2, lanes 1

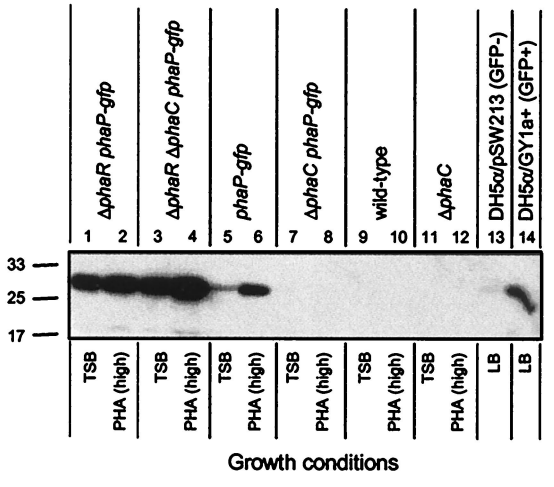

FIG. 2. Immunoblots of GFP in *R. eutropha* Δ*phaR phaP-gfp*, Δ*phaR* Δ*phaC phaP-gfp*, and control strains. Proteins were separated by SDS-PAGE and then subjected to immunoblot analysis for detection of GFP. Molecular mass standards are indicated in kilodaltons. Cells from *R. eutropha* cultures were harvested after cultivation for 48 h in TSB or PHA(high) and resuspended in 10 μl at an OD_{600} of 0.4 for analysis. 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 also included as positive and negative controls, respectively (24-h cultivation in LB plus tetracycline, resuspended in 10 μl at an OD_{600} of 1.0 for analysis). Culture OD_{600} measurements were as follows: lane 1, 9.0; lane 2, 2.7; lane 3, 9.3; lane 4, 1.5; lane 5, 9.2; lane 6, 4.8; lane 7, 9.7; lane 8, 1.5; lane 9, 8.8; lane 10, 14; lane 11, 9.2; lane 12, 5.0; lane 13, 4.2; lane 14, 4.0.

to 4), implying that in the Δ*phaR* background PhaP is synthesized independently of PHB production and thus that PhaR specifically regulates PhaP synthesis. This interpretation is based on the assumption that if cells express a system specifically to degrade PhaP that such a system would not degrade GFP. Importantly, the *phaP-gfp* strain, but not the Δ*phaC phaP-gfp* strain, expressed GFP (Fig. 2, lanes 5 to 8), a finding consistent with our previous observation that in *phaR*$^+$ strains PhaP synthesis is strictly dependent on PHB production (26). Controls confirmed the specificity of the GFP antibody (Fig. 2, lanes 9 to 14).

PhaR directly links PhaP accumulation to the presence of PHB in cells. We proceeded to test whether PhaR is sufficient to regulate PhaP synthesis in a heterologous system. Plasmids containing *phaP* (pGY4+), *phaR* (pGY104−), and *phaP* and *phaR* (pGY105), were constructed from the broad-host-range plasmid pSW213. pAeT41, which contains the *R. eutropha phaCAB* operon and which thus confers upon *E. coli* the ability to produce PHB, has been constructed previously from ColE1-based pUC18. DH5α strains carrying various combinations of the pSW213- and pUC18-based plasmids, and thus varying in their genetic capacities to produce PhaR, PhaP, and PHB, were generated. Strains were cultivated in LB medium and LB medium plus 2% glucose, and culture OD_{600} and PhaP immunoblot analyses were conducted. LB medium was used to prevent PHB production in DH5α/pAeT41 strains (PHB ≤ 0.1% cell dry weight [cdw] at 24 h) and LB 2% glucose was used to promote PHB production (PHB ≥ 60% cdw at 24 h).

DH5α/pGY4+ pUC18 and DH5α/pGY4+ pAeT41 (analogous to *R. eutropha* Δ*phaR* Δ*phaC* and Δ*phaR* strains, respectively) expressed PhaP constitutively (Fig. 3A, lanes 5 to 10). DH5α/pGY105 pUC18 (analogous to the *R. eutropha* Δ*phaC* strain) did not express PhaP (Fig. 3A, lanes 11 to 13; Fig. 3B, lanes 5 to 7). DH5α/pGY105 pAeT41 (analogous to the *R. eutropha* wt strain) expressed PhaP in a manner dependent on PHB production (Fig. 3A, lanes 14 to 16; Fig. 3B, lanes 8 to 10). Specifically, PhaP accumulated to low levels during cultivation in LB (Fig. 3B, lane 8, for which the 0-h time point corresponds to 24-h starter culture) and to high levels during cultivation in LB medium plus 2% glucose (Fig. 3B, lanes 9 to 10). As expected, strains that lack *phaP* (DH5α/pSW213 and DH5α/pGY104− strains) yielded no signal for PhaP (Fig. 3B, lanes 11 to 14). These results demonstrate reconstitution of the PhaR/PhaP regulatory system in a heterologous system and strongly suggest that PhaR regulates PhaP accumulation directly, by negatively regulating PhaP synthesis and coupling PhaP accumulation to PHB production.

There are two caveats that could potentially affect this interpretation. First, pAeT41 includes the first 132 bp of the *phaR* ORF in addition to the *phaCAB* genes and thus could potentially express a truncated but active version of PhaR. However, DH5α/pGY4+ pUC18 and DH5α/pGY4+ pAeT41 accumulated PhaP to approximately the same levels during cultivation in LB (Fig. 3A, lanes 5 and 8, 0-h time point), implying that if such a truncated protein is expressed, it is inactive. Second, pGY4+ contains *phaP* cloned downstream of, and in the same orientation as, the *lac* promoter and thus *phaP* could potentially be transcribed from this promoter. However, two points argue against this possibility. First, transcription from the *lac* promoter is repressed in DH5α. Second, DH5α strains carrying pGY4+ or pGY4− (a plasmid identical to pGY4+ except for the orientation of the 1.5-kb *phaP* fragment) expressed PhaP to similar levels from both plasmids (data not shown), implying that in the absence of PhaR the *phaP* promoter functions constitutively in *E. coli*.

PhaR promotes PHB production in *R. eutropha*. To test whether PhaR affects PHB production, *R. eutropha* wt and Δ*phaR* strains were cultivated under two sets of conditions that promote PHB production: PHA(high) and LB medium plus 2% fructose. These growth conditions were chosen to allow comparisons between *R. eutropha* and *E. coli*. PHA(high) is a standard growth medium for PHB production in *R. eutropha* (16, 27), and LB medium plus 2% fructose is analogous to LB medium plus 2% glucose, a standard growth medium for PHB production in *E. coli* (10, 23). Strains were cultivated in TSB starter cultures, transferred to PHA(high) and LB medium plus 2% fructose treatment cultures, both at an initial OD_{600} of 1.0 (high titer) and at OD_{600} of 0.1 (low titer), and cultivated for 3 days. The two inoculation conditions were used to test whether any effect of deletion of *phaR* might depend on the initial density of cells in cultures. PHB production was measured over a time course. The Δ*phaP* and Δ*phaR* Δ*phaP* strains were also characterized to test the effect of PhaP on PHB production.

The Δ*phaR* strain accumulated PHB to ≤70 and ≤55% the level of the wt strain at the peak of PHB accumulation during cultivation in PHA(high) and LB medium plus 2% fructose, respectively (Fig. 4A, C, E, and G). PHB levels rose throughout cultivation in PHA(high) (Fig. 4A to D), whereas PHB levels rose and then fell during cultivation in LB plus 2% fructose (Fig. 4E to H). PHB reached higher levels for high-titer inoculations (except for the wt strain cultivated in LB medium plus 2% fructose) and deletion of *phaR* had a more dramatic effect on PHB production for low-titer inoculations (Fig. 4A, B, E, and F versus Fig. 4C, D, G, and H). These observations suggest that overexpression of PhaP may have a more dramatic negative effect on PHB production with decreasing titers of inoculation. Deletion of *phaR* clearly leads to decreased PHB yields, but it is not clear whether PhaR promotes PHB synthesis, prevents PHB degradation, or both. PhaP was present at 2.5- to 8.5-fold-higher levels in Δ*phaR* cells than in wt cells throughout cultivation [PHA(high)/high-titer inoculation] (Fig. 5), suggesting that PhaP overexpression may contribute to the PHB production defect of the Δ*phaR* strain. However, the Δ*phaR* Δ*phaP* strain exhibited a more severe defect in PHB production than the Δ*phaP* strain, particularly during cultivation under high-titer conditions (Fig. 4A, B, E, and F), indicating that PhaR also promotes PHB production by a PhaP-independent mechanism.

PhaP, but not PhaR, promotes PHB production in a heterologous system. We proceeded to test whether PhaR and PhaP also affect PHB production in *E. coli*. DH5α/pSW213 pAeT41 and DH5α/pGY104− pAeT41 strains (analogous to *R. eutropha* Δ*phaR* Δ*phaP* and Δ*phaP* strains, respectively) exhibited similar yields of PHB (Fig. 6A and B), indicating that PhaR does not directly affect PHB production in the absence of PhaP. DH5α/pGY4+ pAeT41 and DH5α/pGY105 pAeT41 (analogous to *R. eutropha* Δ*phaR* and wt strains, respectively) exhibited higher yields of PHB than DH5α/pSW213 pAeT41 and DH5α/pGY104− pAeT41, indicating that PhaP promotes

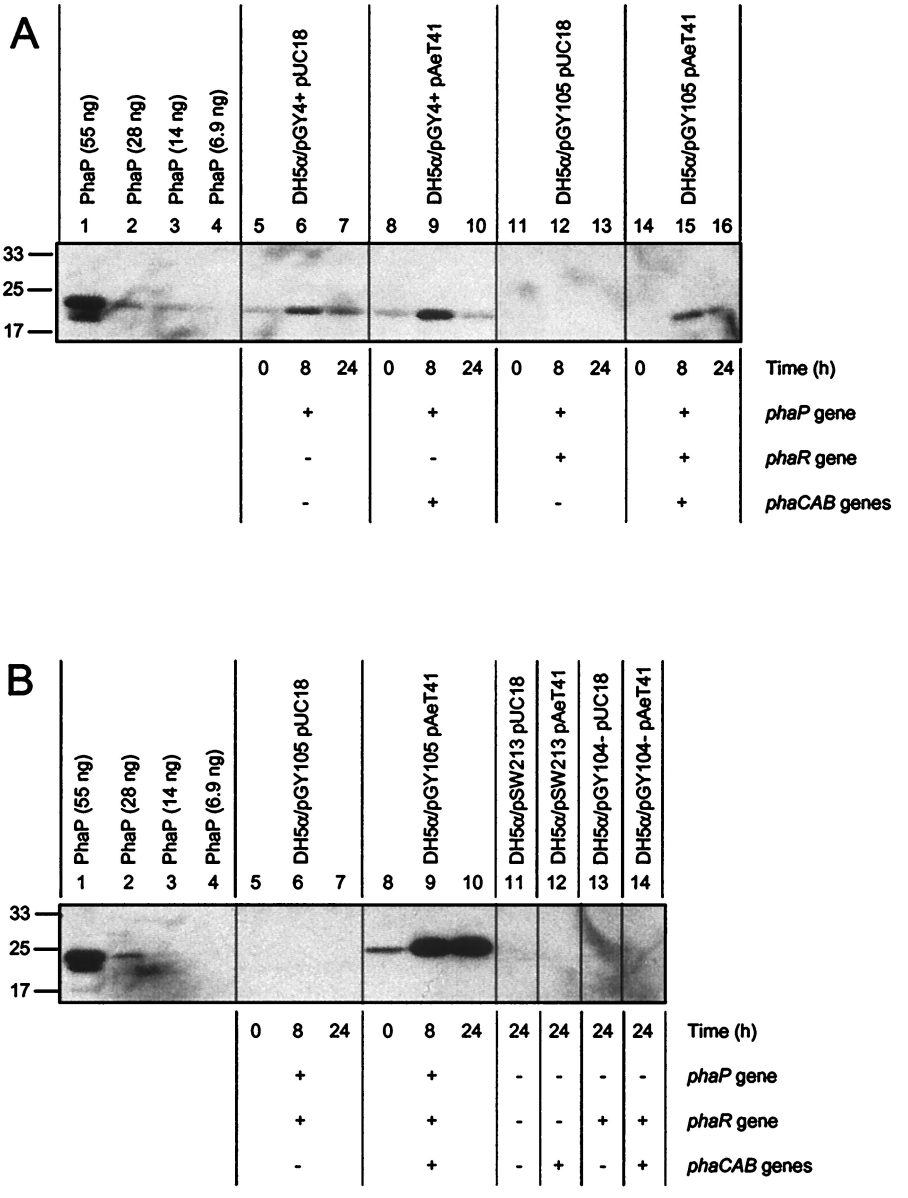

FIG. 3. Immunoblots of PhaP in cultures of *E. coli* strains that carry the *R. eutropha phaP*, *phaR*, and/or *phaCAB* genes. Proteins were separated by SDS-PAGE and then subjected to immunoblot analysis for PhaP. Purified PhaP was included as a control on both gels. Molecular mass standards are indicated in kilodaltons. Cells were harvested after cultivation of strains in LB medium plus 2% glucose(Ap Tc) at various time points (0, 8, and 24 h). (A) Samples correspond to cells from 10 μl of culture diluted to an OD_{600} of 0.20. Note that lanes 1 to 10 and lanes 11 to 16 correspond to different gels. Culture OD_{600} measurements were as follows: lane 5, 0.9; lane 6, 4.1; lane 7, 3.3; lane 8, 0.9; lane 9, 11; lane 10, 33; lane 11, 0.9; lane 12, 4.2; lane 13, 4.4; lane 14, 0.9; lane 15, 14; lane 16, 32. (B) Samples correspond to cells from 10 μl of culture diluted to an OD_{600} of 2.0. Culture OD_{600} measurements were as follows: lane 11, 4.2; lane 12, 21; lane 13, 4.4; lane 14, 18.

PHB production in this system (Fig. 6A and B). DH5α/pGY4+ pAeT41 and DH5α/pGY105 pAeT41 also accumulated PHB to approximately the same levels by 3 days, suggesting that PhaR-mediated regulation of PhaP accumulation does not affect overall PHB production. Interestingly, at 5 h the DH5α/pGY4+ pAeT41 strain, which expresses PhaP constitutively, exhibited low levels of PHB relative to the other three DH5α/pAeT41 strains. Analyses of PHB production by the DH5α/pAeT41 strains at early time points in an independent experiment confirmed this observation (Fig. 6C and D). Apparently, overexpression of PhaP transiently delays or decreases PHB production, implying that PhaR-mediated regulation of PhaP may be important, but only at the onset of PHB synthesis. It is not clear how these results relate to *R. eutropha*, since *R. eutropha* cultures were always inoculated with cells that already contained PHB (PHB > 5% cdw).

DISCUSSION

Our results demonstrate that PhaR is a negative regulator of PhaP synthesis that directly couples PhaP synthesis to PHB production. The simplest model for PhaR regulation of PhaP

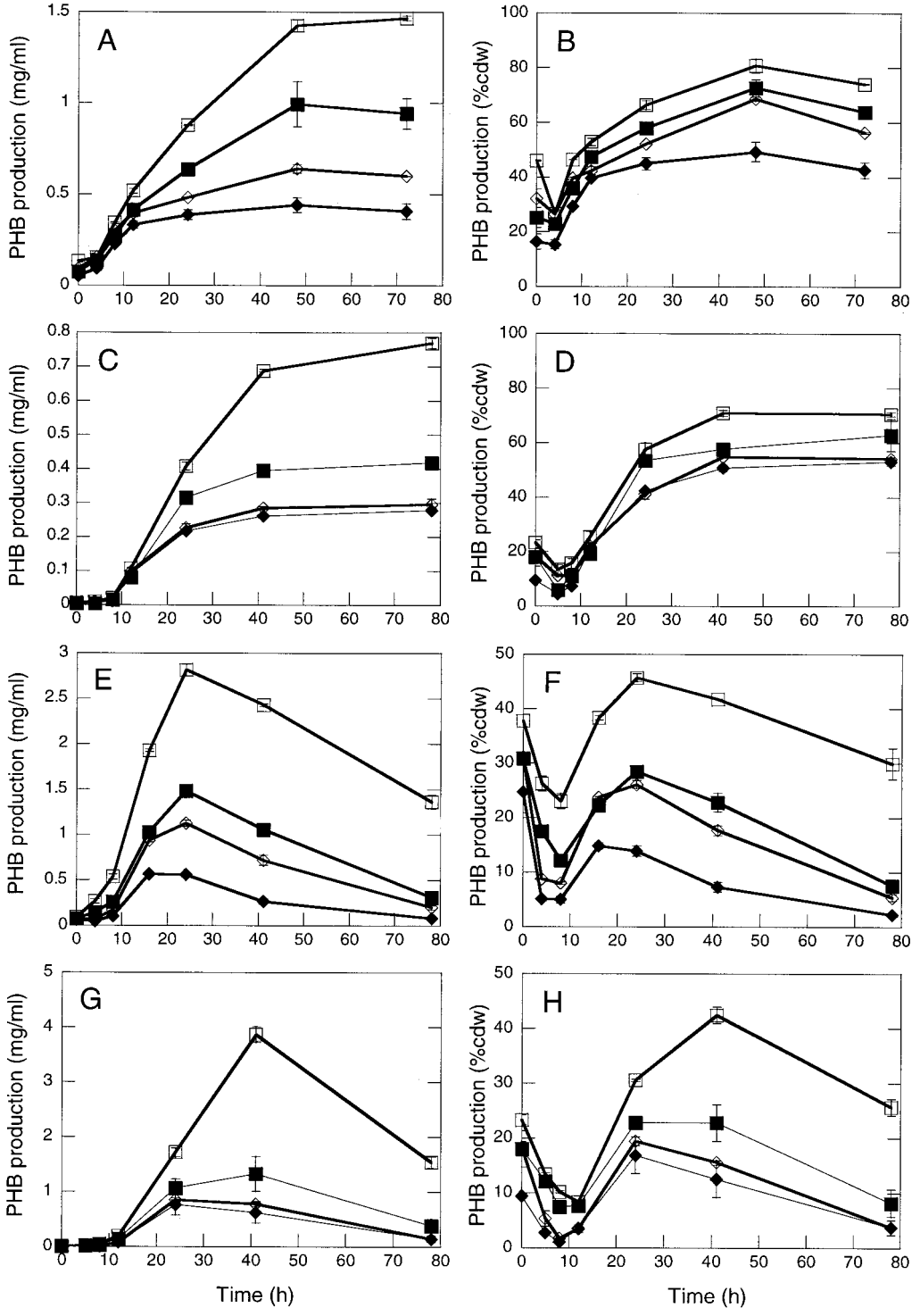

FIG. 4. Comparison of PHB production for *R. eutropha* wt, Δ*phaR*, Δ*phaP*, and Δ*phaR* Δ*phaP* strains. Symbols: open squares, wt strain; closed squares, Δ*phaR* strain; open diamonds, Δ*phaP* strain; solid diamonds, Δ*phaR* Δ*phaP* strain. Growth media correspond to PHA(high) (A to D) and LB medium plus 2% fructose (E and F). Inoculation conditions correspond to high titer (A, B, E, and F) and low titer (C, D, G, and H). PHB production is expressed as mass PHB per volume culture (A, C, E, and G) and as PHB % cdw (B, D, F, and H). Datum points represent average value for two independent cultures. Error bars indicate the standard deviation.

accumulation is that PhaR binds a regulatory sequence upstream of the *phaP* ORF and blocks its transcription. The simplest mechanism by which PhaR may couple PhaP synthesis to PHB production is that, upon the onset of PHB synthesis in cells, PhaR is titrated from *phaP*, possibly by binding to PHB or else by interacting with a factor that is present in *R. eutropha* and *E. coli* cells only after these cells have begun to synthesize PHB. PhaP synthesis then occurs to an extent that is deter-

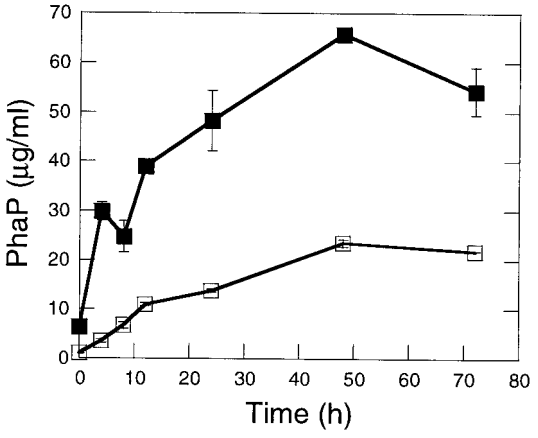

FIG. 5. Quantitation of PhaP in *R. eutropha* wt and Δ*phaR* strains, as cultivated in PHA(high) by high-titer inoculation and as measured over a time course of 72 h. Symbols: open squares, wt strain; solid squares, Δ*phaR* strain. All datum points represent the average value for two independent cultures. Error bars indicate the standard deviation. Samples correspond to the cultures of wt and Δ*phaR* strains in Fig. 4A and B.

mined by the degree of PhaR titration and the availability of nutrients. This model accounts for the observations that in *R. eutropha* wt cells PhaP synthesis is strictly dependent on PHB synthesis, that in *R. eutropha* Δ*phaR* cells PhaP is synthesized constitutively, and that in *E. coli* cells addition of the *phaCAB*, *phaR*, and *phaP* genes is sufficient to reconstitute the PhaR/PhaP regulatory system.

Our results suggest that PhaR promotes PHB synthesis in *R. eutropha* by regulating the expression of PhaP and one or more additional proteins, thus functioning by PhaP-dependent and PhaP-independent pathways. For the PhaP-dependent pathway, PhaR represses synthesis of PhaP until after the onset of PHB synthesis, thus preventing diversion of metabolites toward production of excess PhaP and/or preventing PhaP from interfering with the initiation of PHB synthesis. This would account for the observations that *E. coli phaCAB*$^+$ strains that overexpress PhaP exhibit a defect in PHB synthesis at the onset of PHB synthesis and that *R. eutropha phaR* mutants exhibit more severe defects in PHB production for low-titer versus high-titer inoculations. For the PhaP-independent pathway, PhaR represses the synthesis of one or more additional proteins that may interfere with PHB production when expressed at inappropriate times or levels or that may promote utilization

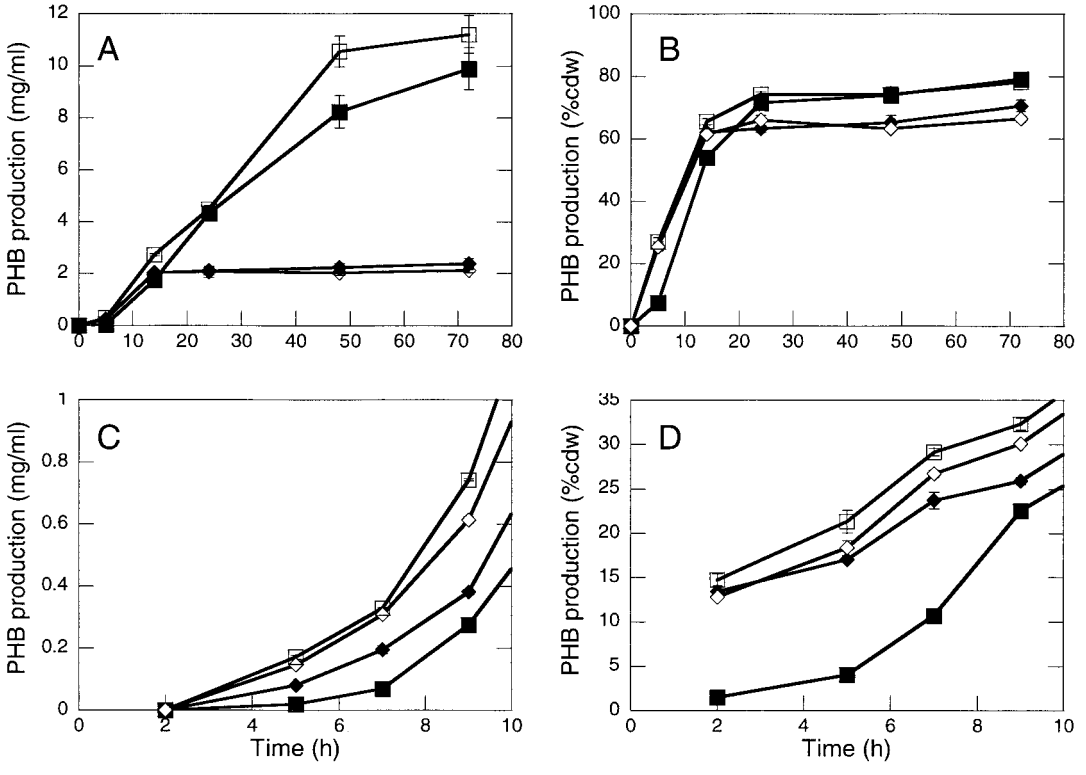

FIG. 6. Comparison of PHB production for *E. coli* strains that carry the *R. eutropha phaCAB* genes and that vary with respect to their capacity to produce PhaP and PhaR. Symbols: open squares, DH5α/pGY105 pAeT41; solid squares, DH5α/pGY4+ pAeT41; open diamonds, DH5α/pGY104− pAeT41; solid diamonds, DH5α/pSW213 pAeT41. The growth medium corresponds to LB medium plus 2% glucose(Ap Tc). Cultures were inoculated at an initial OD_{600} of 0.15. The data represent two independent cultivation experiments. The datum points represent average values for two independent cultures in a given experiment. The error bars indicate the standard deviation. Results for panels A and B and from panels C and D are from independent experiments. (A) PHB accumulation (milligrams/milliliter of culture). (B) PHB accumulation (% cdw). (C) PHB production (milligrams/milliliter of culture). DH5α/pAeT41 strains accumulated PHB to the following amounts after 105 h of cultivation: pGY105, 8.7 ± 0.13; pGY4+, 8.9 ± 0.47; pGY104−, 4.3 ± 0.071; and pSW213, 4.2 ± 0.25. (D) PHB production (% cdw). DH5α/pAeT41 strains accumulated PHB to the following amounts after 105 h of cultivation: pGY105, 80% ± 0.78%; pGY4+, 81% ± 1.5%; pGY104−, 72% ± 1.4%; and pSW213, 74% ± 0.90%.

of PHB under inappropriate conditions. This would account for the observations that Δ*phaR* Δ*phaP* mutants exhibit more severe defects in PHB production than Δ*phaP* mutants and that *E. coli phaCAB*$^+$ cells exhibit the same final yields of PHB independent of *phaR*.

Several other groups have reported on PhaR/PhaP and other PHA regulatory systems recently. Maehara et al. (13) transferred *P. denitrificans phaR* and *phaP* into *E. coli* and demonstrated that the presence of *phaR* dramatically decreased expression of *phaP*. Maehara et al. (12) also recently reported that PhaR represses *phaP* expression in vitro. However, in contrast to *R. eutropha* PhaR/PhaP, *P. denitrificans* PhaR did not completely block *phaP* expression in *E. coli*, even in cells that cannot produce PHA (13). Thus, while *P. denitrificans* PhaR negatively regulates expression of *phaP*, it remains to be determined whether it also couples PhaP synthesis to PHB production. Prieto et al. (19) have proposed a model regarding another PHA-related regulatory system, PhaF-mediated regulation of protein expression in *P. oleovorans* GPo1, that is similar to the *R. eutropha* PhaR/PhaP system. According to the model, PhaF binds DNA, responds to PHA or factors present during PHA synthesis, and regulates the expression of a granule-associated protein (9, 19). However, PhaF and PhaR are not homologs and, unlike PhaR, PhaF also regulates expression of PHA synthase (19). Thus, the systems are distinct. Povolo and Cadela (18) have demonstrated that deletion of the *S. meliloti phaR* homolog *aniA* results in significant changes in carbohydrate polymer production and nitrogenase activity, in addition to a decrease in PHB levels. The observation that many metabolic processes are affected by the *aniA* mutation is consistent with the idea that PhaR regulates the expression of proteins in addition to phasins.

Comparisons of the properties and yields of a variety of PHAs, as produced by *R. eutropha* wt, *phaR*Δ, *phaP*Δ, and *phaR*Δ *phaP*Δ strains and *E. coli* strains engineered to express PhaR, PhaP, and PHA biosynthetic enzymes, are providing new insights into the role and specificity of phasins in PHA production. A long-range goal is that the PhaR/PhaP regulatory system and PhaP may eventually serve as tools to accomplish the production of novel PHAs in an economically competitive manner.

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