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Poly-β-Hydroxybutyrate Biosynthesis in the Facultative Methylotroph *Methylobacterium extorquens* AM1: Identification and Mutation of *gap11*, *gap20*, and *phaR*

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Methylobacterium extorquens **AM1, a serine cycle facultative methylotroph, accumulates poly-β-hydroxybutyrate (PHB) as a carbon and energy reserve material during growth on both multicarbon- and single-carbon substrates. Recently, the identification and mutation of the genes involved in the biosynthesis and degradation of PHB have been described for this bacterium, demonstrating that two of the genes of the PHB cycle (*phaA* and *phaB*) are also involved in C_1 and C_2 metabolism, as part of a novel pathway for glyoxylate regeneration in the serine cycle (N. Korotkova and M. E. Lidstrom, J. Bacteriol. 183:1038-1046, 2001; N. Korotkova, L. Chistoserdova, V. Kuksa, and M. E. Lidstrom, J. Bacteriol. 184:1750-1758, 2002). In this work, three new genes involved in PHB biosynthesis in this bacterium have been investigated via mutation and phenotypic analysis: *gap11*, *gap20*, and *phaR*. We demonstrate that *gap11* and *gap20* encode two major granule-associated proteins (phasins) and that mutants with mutations in these genes are defective in PHB production and also in growth on C_2 compounds, while they show wild-type growth characteristics on C_1 or multicarbon compounds. The *phaR* mutant shows defects in both PHB accumulation and growth characteristics when grown on C_1 compounds and has defects in PHB accumulation but grows normally on C_3 and C_4 compounds, while both PHB accumulation and growth rate are at wild-type levels during growth on C_2 compounds. Our results suggest that this phenotype is due to altered fluxes of acetyl coenzyme A (CoA), a major intermediate in C_1, C_2, and heterotrophic metabolism in *M. extorquens* AM1, as well as the entry metabolite for the PHB cycle. Therefore, it seems likely that PhaR acts to control acetyl-CoA flux to PHB in this methylotrophic bacterium.**

Poly-β-hydroxybutyrate (PHB) and other polyhydroxyalkanoates (PHAs) are accumulated by many bacteria as an energy and carbon reserve material (1). While the enzymology and the genetics of PHA biosynthesis have been extensively studied for a number of organisms and are now well understood, less is known about the regulation of this process. A series of recent studies have been undertaken to unravel the mechanisms by which PHA biosynthesis is controlled in a number of bacteria with different modes of metabolism, and the emerging patterns seem to be complex. So far, two major types of PHA accumulation regulators have been investigated: (i) phasins, the proteins that bind to PHA granules and promote PHA synthesis, and (ii) regulators having an effect ranging from regulating phasin biosynthesis to more complex, pleiotropic effects. These are known under two different names, PhaR and AniA (11, 12, 28, 29, 46). In addition, two different types of transcriptional regulators have been reported elsewhere to be involved in PHB production in *Azotobacter vinelandii* (4, 45).

So far, phasins appear to be present in all PHA-synthesizing bacteria, and even though they generally are not conserved in sequence and seem to be species specific, they are believed to fulfill the same function, binding to PHA granules and promoting PHA synthesis, in a manner still poorly understood (19). It has been shown elsewhere that phasins stabilize the PHA granules, prevent them from coalescing, and control the granule size (15, 18, 19, 34). In accordance with one hypothesis, phasins must bind directly to PHA and possibly to the PHA-cycling enzymes, PHA synthase and/or PHA depolymerase (15, 18), while in accordance with a second hypothesis, they may bind to the granules themselves and prevent binding of proteins not related to PHA metabolism (18, 19). The second type of regulator, now identified in many bacteria and designated PhaR, was first identified in close proximity to a gene encoding a phasin in *Paracoccus denitrificans* and implicated in its negative regulation (28, 29). Negative regulation of a phasin by a PhaR homolog was also reported for *Ralstonia eutropha*; however, in the latter case, PhaR also influenced PHB production in a phasin-independent fashion, which remains unknown (46). In *Sinorhizobium meliloti*, a homolog of PhaR was called AniA, because of its expression during anaerobic growth. An even more complex function was suggested for this protein, in partitioning of carbon flow in cells, affecting not only PHB production but also production of extracellular polymeric substances and nitrogen fixation (36). AniA was also investigated in another rhizobial species, *Rhizobium etli*, and suggested to play a role not only in PHB production but also as a global regulator of protein expression and of carbon and energy fluxes, based on the pleiotropic effect of mutation in AniA on metabolism of this bacterium (11, 12).

Methylobacterium extorquens AM1 accumulates PHB and forms PHB granules like other PHB-producing bacteria (13, 14). PHB content in *M. extorquens* AM1 cells varies depending

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on growth substrate (23). Genes and enzymes participating in the biosynthesis and degradation of PHB have recently been identified in *M. extorquens* AM1, and their roles in PHB cycling have been studied via mutation (23). Mutants with mutations in the first two enzymes for PHB synthesis from acetyl coenzyme A (CoA), β-ketothiolase (PhaA) and NADPH-linked acetoacetyl-CoA reductase (PhaB), revealed not only a PHB deficiency but also a deficiency in growth on C_1 and C_2 compounds. It was determined that this growth deficiency was due to the participation of these genes in the novel pathway for regeneration of glyoxylate from acetyl-CoA in the serine cycle (23, 24). Thus, in this methylotrophic bacterium, the PHB biosynthesis pathway overlaps with the central metabolic pathways for C_1 and C_2 assimilation. Such a central placement of the PHB cycle in the metabolic network of *M. extorquens* AM1 would suggest specific mechanisms for regulating synthesis and degradation of PHB depending on growth substrate-specific metabolic modes. In this work we identify the *phaR* gene in *M. extorquens* AM1 and investigate the effect of mutation in this gene on growth and PHB production on different substrates. We also identify two proteins associated with PHB granules in this organism, Gap11 and Gap20.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and DNA manipulations. *Escherichia coli* strains DH5α (Gibco-BRL, Rockville, Md.), S17-1 (40), and BL21(DE3) (Novagen, Inc., Madison, Wis.) used in the study were grown in Luria-Bertani medium in the presence of appropriate antibiotics as described by Maniatis et al. (30). *M. extorquens* AM1 was grown in the minimal medium described previously (16). Succinate (20 mM), pyruvate (20 mM), methanol (100 mM), or ethylamine (20 mM) was used as a substrate. The antibiotic concentrations used for *M. extorquens* AM1 were as follows: tetracycline, 10 $\mu g\ ml^{-1}$; kanamycin, 100 $\mu g\ ml^{-1}$; and rifamycin, 50 $\mu g\ ml^{-1}$. Growth responses of the *phaR* mutant were tested in methanol-containing medium supplemented with acetate (0.1 to 0.2 mM). For methanol induction, strains were grown on succinate to mid-logarithmic phase, pelleted, and incubated in methanol (100 mM)-containing medium overnight with shaking at 30°C. The following cloning vectors were used: pUC19 (Amersham Pharmacia Biotech, Inc. Piscataway, N.J.) for cloning and subcloning, pAYC61 (5) as a suicide vector, pRK2013 (10) as a helper plasmid, pCR2.1 (Invitrogen, Carlsbad, Calif.) for cloning of PCR products, pET21d(+) (Novagen, Inc.) as an expression vector, and pCM130 (31) as a promoter probe vector. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, and ligation were carried out as described by Maniatis et al. (30). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure of Saito and Miura (38).

Gene amplification and cloning. Data from the *M. extorquens* AM1 genome project (http://vixen.microbiol.washington.edu/) were used for designing primers specific for putative genes of *M. extorquens* AM1 encoding PhaR, Gap11, and Gap20 (primer sequences may be provided on request). The identity of the amplified DNA fragments was confirmed by sequencing from both strands performed by the Department of Biochemistry, University of Washington Sequencing Facility.

Mutant generation. Insertion mutations in *phaR*, *gap11*, and *gap20* were generated in vitro with the Km^r gene cartridge as described earlier (5–8). The sites used for inserting the Km^r gene cartridge were as follows: the *Bsg*I site in the first half of *phaR*, the *Pst*I site in the putative ribosome-binding region of *gap11*, and the *Hinc*II site in the first half of *gap20*. Triparental or biparental matings between *E. coli* carrying the respective donor plasmids and *M. extorquens* AM1 were performed overnight on nutrient agar at 30°C. Cells were then washed with sterile medium and plated on selective medium at appropriate dilutions. In triparental matings, pRK2013 (10) was used as a helper plasmid. Rifamycin was used for *E. coli* counterselection. In all cases, mutants were selected on succinate plates in the presence of kanamycin, and Km^r colonies were checked for their resistance to tetracycline. Tc^s colonies were chosen as potential double-crossover recombinants, while Tc^r colonies were assumed to be single-crossover recombinants. The identity of the double-crossover mutants was confirmed by diagnostic PCR.

Computer analysis. Sequence comparisons and analysis were performed with BLAST and search tools available on the *M. extorquens* AM1 genome website (http://vixen.microbiol.washington.edu/) and also tools and programs available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Motif searches were performed with two different programs for helix-turn-helix motif prediction available at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_hth.html and at http://www.es.embnet.org/Services/MolBio/hth.html.

Enzyme assays. Activities of β-ketothiolase and acetoacetyl-CoA reductase were determined as described previously (39), and the activity of catechol 2,3-dioxygenase was assayed as described by Kataeva and Golovleva (21) in *M. extorquens* AM1 crude extracts obtained by passing cells through a French pressure cell at 1.2×10^8 Pa, followed by centrifugation for 10 min at approximately $15{,}000 \times g$. All measurements were done at room temperature (26°C) in a total volume of 1 ml. Spectrophotometric methods (20, 43) were used for protein determination. All assays were done in triplicate, and results agreed within ±15%.

Determination of distribution of ^{14}C from [2-^{14}C]acetate between CO_2 and biomass. Succinate-grown cells were harvested by centrifugation (5 min, $6{,}000 \times g$), washed, and resuspended in a medium containing methanol (100 mM). Cells were incubated with shaking at 30°C for 1 h for induction, after which [2-^{14}C]acetate (0.1 mM, 0.2 μCi per reaction vial) was added and samples were taken at 10-min intervals. The percentage of total radioactivity in cell biomass versus CO_2 was determined and calculated as described previously (24).

PHB analysis. PHB concentration in bacterial cells was determined by a gas chromatographic method (2). A model GC-14A capillary gas chromatograph (Shimadzu, Kyoto, Japan) with an AT-WAX capillary column (0.53 mm by 10 m; 1.2-μm film thickness; Alltech, Deerfield, Ill.) and a flame ionization detector was used. The flow rate of helium carrier gas was 2.7 ml/min. The initial column temperature of 60°C was held for 2 min, and then the temperature was increased by 5°C/min up to 160°C.

PHB granule isolation. PHB granules were isolated on a sucrose gradient by the method of Föllner and colleagues (13). The pelleted granules were resuspended in gel loading buffer. After 5 min of incubation at 100°C, the granule-associated proteins (GAPs) were separated by gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) according to the method of Laemmli (25) with 8 to 12% gradient acrylamide gels (Bio-Rad Laboratories, Hercules, Calif.).

***xylE* fusion construction.** Putative promoter regions for *phaA* (650 bp), *phaB* (450 bp), *phaC* (740 bp), *phaR* (650 bp), *gap11* (450 bp), and *gap20* (620 bp) were amplified by PCR and cloned into the pCR2.1 vector. These fragments covered the entire intergenic regions, contained the 5′ termini of the genes whose promoters were being tested, and also overlapped with the neighboring genes by their 5′ or 3′ termini. The resulting fragments were subsequently cut out with appropriate restriction enzymes and inserted into the promoter probe vector pCM130 (31), upstream of a promoterless *xylE* gene.

Electrophoretic mobility shift assays. An 0.65-kb PCR product containing the entire *phaR* gene and its putative ribosome-binding site was cloned into pCR2.1, redigested with *Xba*I-*Bam*HI, and cloned into pET21d(+) in the orientation allowing transcription from the T7 promoter. The resulting plasmid was transferred into the expression strain *E. coli* BL21(DE3). The resulting *E. coli* strain was grown in Luria-Bertani medium supplemented with 100 μg of ampicillin/ml at 37°C with shaking at 350 rpm to an optical density of 0.4 to 0.6 at 600 nm. Expression of PhaR was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by a 4-h incubation. Cells were collected by centrifugation, resuspended in 3 ml of 50 mM sodium phosphate (pH 7.5)–200 mM KCl, and disrupted with a French pressure cell at 1.2×10^8 Pa, followed by centrifugation for 20 min at approximately $15{,}000 \times g$. The supernatant was used for gel shift assays.

Target DNA fragments of about 400 to 500 bp were amplified by PCR and end labeled by phosphorylation with T4 DNA kinase (Promega, Madison, Wis.). The cell extract containing PhaR (8 to 10 μg) was incubated with the labeled DNA for 20 min at room temperature in the following gel shift binding buffer (Promega): 5 mM Tris HCl (pH 7.5)–50 mM NaCl–1 mM $MgCl_2$–0.5 mM dithiothreitol–0.5 mM EDTA–4% glycerol–0.05 μg of poly(dI-dC)/ml. Cell extract of *E. coli* containing no PhaR was used as a control. After incubation, the mixtures were loaded on a Novex 6% retardation gel (Invitrogen) or a 6% nondenaturing acrylamide gel in 0.5× Tris-borate-EDTA and electrophoresed at 300 V. Gels were subsequently dried and exposed to X-ray film (Kodak).

Nucleotide sequence accession numbers. The nucleotide sequences of fragments containing *phaR*, *gap11*, and *gap20* have been deposited with GenBank under accession no. AF287907, AF442748, and AF442749, respectively.

RESULTS

Identification of *phaR*, *gap11*, and *gap20*. In a previous study, *phaA* and *phaB* were identified as encoding, respectively, β-ketothiolase and NADPH-linked acetoacetyl-CoA reductase on a single 6.1-kb fragment of the *M. extorquens* AM1 chromosome (23). *orf3* was found upstream of *phaA* (23). The potential polypeptide translated from this gene showed high identity (up to 56%) with proteins translated from regions containing genes for PHB biosynthesis in a number of proteobacteria (3, 11, 12, 36). For rhizobia, this gene has been designated *aniA* and implicated in a complex regulation of carbon flow in these bacteria (12, 36). Orf3 also showed lower identities with PhaR proteins in *P. denitrificans* and *R. eutropha*, which have been implicated elsewhere in negative regulation of GAPs in these organisms (28, 29, 46). Therefore, we selected *orf3* for further analysis, as a potential regulator of PHB synthesis, and redesignated it *phaR*.

GAPs, also called phasins, have been implicated elsewhere in regulating PHB accumulation (18, 22, 37, 46). To identify genes potentially encoding GAPs in *M. extorquens* AM1, we used the N-terminal amino acid sequences for two GAPs, of 11 and 20 kDa (GA11 and GA20, respectively), previously identified for a similar strain, *M. extorquens* MB371 (14), as queries to search the genomic database of *M. extorquens* AM1 (http://vixen.microbiol.washington.edu/). We identified the two respective genes and designated them *gap11* and *gap20*. *gap11* and *gap20* are not linked on the chromosome to each other or to other genes known to be involved in PHB biosynthesis in *M. extorquens* AM1. The molecular masses deduced for the polypeptides translated from *gap11* and *gap20* (12.8 and 19.1 kDa, respectively) were in agreement with the molecular masses experimentally determined for GA20 and GA11 from *M. extorquens* MB371, and the N-terminal amino acid sequences translated from *gap11* and *gap20* were identical to the experimentally determined sequences of GA11 and GA20 (13, 14). Gap20 and Gap11 showed high identity to each other (42%), and they revealed similarity with GAPs from *Zoogloea ramigera* (17) and unknown proteins from PHB-synthesizing α-proteobacteria *Rhodopseudomonas palustris*, *Mesorhizobium loti* MAFF303099, and *S. meliloti* (http://jgi.doe.gov/JGI_microbial/html/index.html).

Gap11, Gap20, and PhaR were inactivated by site-directed mutagenesis to test their role in PHB accumulation and general metabolism of *M. extorquens* AM1.

Mutations in Gap11 and Gap20 affect PHB accumulation and growth of *M. extorquens* AM1. Mutations in Gap11 and Gap20 were generated as described in Materials and Methods, and the resulting mutants were characterized with respect to PHB accumulation, PHB granule content, and growth characteristics. Four types of growth substrates were tested: a C_1 substrate (methanol), a C_2 substrate (ethylamine), a C_3 substrate (pyruvate), and a C_4 substrate (succinate). Mutation in Gap11 or Gap20 had no effect on growth of *M. extorquens* AM1 on the C_1, C_3, or C_4 substrate, while in both cases growth on the C_2 substrate was altered (Fig. 1). PHB accumulation was also affected in the mutants, and different patterns were observed depending on the growth substrate (Table 1). Lower PHB levels were determined for both mutants grown on methanol, and the effect was more dramatic in the Gap20 mutant. PHB accumulation dropped by about one-half in cells of both mutants grown on ethylamine, while it remained at wild-type level in cells grown on pyruvate. The effect on PHB accumulation during succinate growth was different for the two mutants. The Gap20 mutant accumulated a reduced amount of PHB compared to the wild-type strain, while in the Gap11 mutant PHB accumulation increased about twofold. These data suggest that the two GAPs in *M. extorquens* AM1 must have distinct functions in controlling PHB accumulation and these functions must be different in different metabolic circumstances.

To prove that Gap11 and Gap20 are associated with PHB granules in *M. extorquens* AM1 and to assess and compare their levels, we analyzed PHB granules from wild-type *M. extorquens* AM1 and from the Gap11 and Gap20 mutants. Granules were isolated from methanol- and succinate-grown cultures and subjected to electrophoresis in denaturing conditions. Two major proteins are present in wild-type granules, with molecular masses of approximately 11 and 20 kDa (Fig. 2). In the Gap11 mutant, only the 20-kDa band is present, and in the Gap20 mutant, only the 11-kDa band is seen, confirming that the products of *gap11* and *gap20* are the major GAPs in *M. extorquens* AM1. As seen in Fig. 2, Gap11 and Gap 20 are present at different levels in the granules, with Gap11 being much more abundant than Gap20. These data were also confirmed by measuring expression levels for *gap11* and *gap20*, employing transcriptional fusions to a reporter gene, *xylE*, encoding catechol 2,3-dioxygenase (XylE). As seen from Table 2, the difference in XylE activities in *M. extorquens* AM1 carrying the $P_{gap11:xylE}$ and $P_{gap11:xylE}$ fusions is about 60-fold. These data suggest that *gap11* and *gap20*, in addition to being involved in controlling PHB levels, are themselves subjects of regulation. An obvious candidate as a regulator was the *phaR* gene.

Mutation in *phaR* affects PHB accumulation and growth of *M. extorquens* AM1. PhaR has been implicated previously in playing a role in negative regulation of GAPs (phasins) in some bacteria (i.e., *P. denitrificans* and *R. eutropha*), while for others (rhizobia) a PhaR homolog, AniA, was shown to play a role in global regulation of carbon flow, and mutation in this gene caused highly pleiotropic effects (11, 12, 36). To test the role of PhaR in *M. extorquens* AM1, we generated a null mutation in *phaR* and investigated the mutant phenotype with respect to growth characteristics on various substrates, PHB accumulation during growth on various substrates, and expression of genes involved in PHB biosynthesis. Unlike mutations in the GAPs, the mutation in PhaR had a dramatic effect on growth of *M. extorquens* AM1 on the C_1 compound (Fig. 1A), while growth on the C_2, C_3, and C_4 compounds was not affected (Fig. 1B to D). PHB accumulation was also dramatically affected in the PhaR mutant when grown on the C_1, C_3, and C_4 compounds. However, PHB accumulation on the C_2 compound was at wild-type levels (Table 1). These data suggest that PhaR must be involved in controlling PHB biosynthesis during growth on C_1, C_3, and C_4 compounds, but not on C_2 compounds. The growth defect on methanol is not due to the decreased PHB synthesis per se, as the GAP mutants both had greatly reduced PHB synthesis but normal growth on methanol. In addition, a mutant has been previously described that is

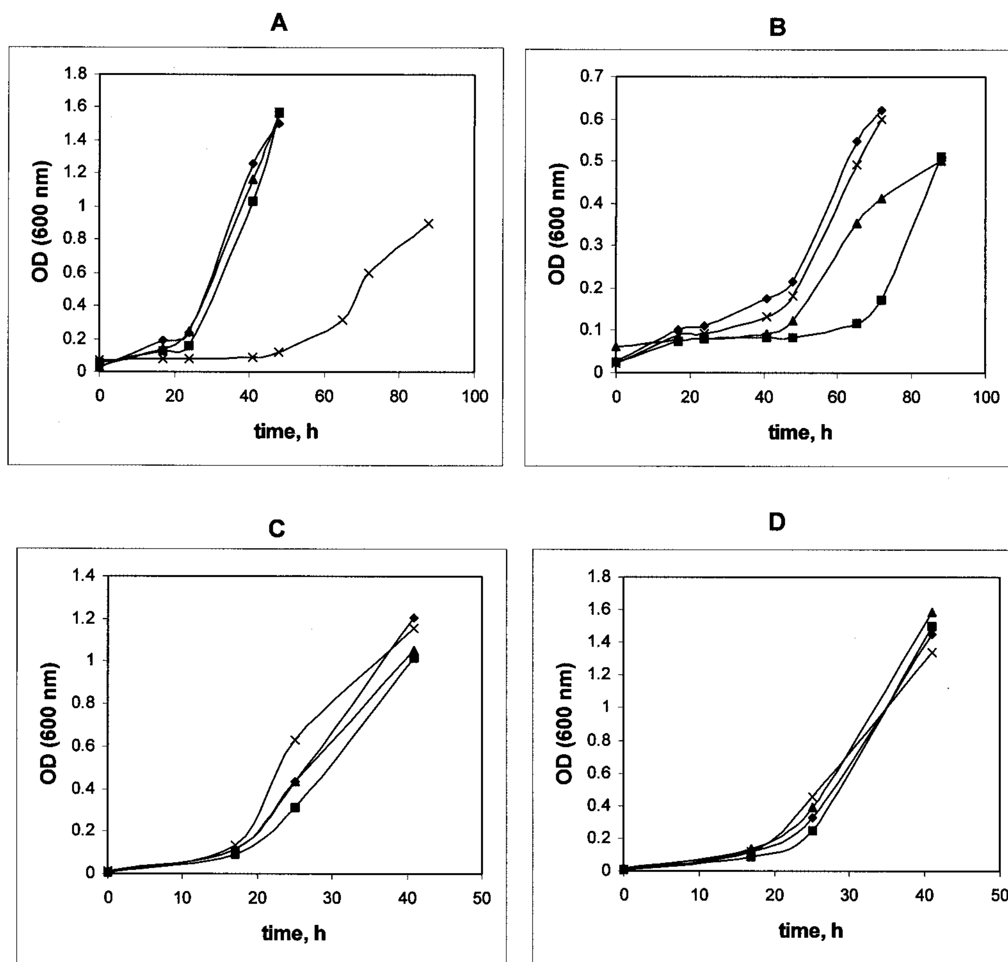

FIG. 1. Growth of *M. extorquens* AM1 and *gap20*, *gap11*, and *phaR* mutants on methanol (A), ethylamine (B), succinate (C), and pyruvate (D). Symbols: diamond, *M. extorquens* AM1; square, *gap11* mutant; triangle, *gap20* mutant; cross, *phaR* mutant. OD, optical density.

unable to synthesize PHB but which grows normally on methanol (23).

Investigation of PhaR as a transcriptional regulator in *M. extorquens* AM1. For *P. denitrificans*, PhaR was shown to specifically bind to chromosomal regions upstream of *phaP* encoding a phasin and to *phaR* and to negatively regulate expression of these genes (29). However, the expression results showed that in *M. extorquens* AM1 PhaR is transcribed at a high level, comparable to that of major metabolic enzymes (see below), an unusual attribute of a transcriptional regulator. It is worth noting that PhaR polypeptides from *M. extorquens* AM1 and *P. denitrificans* share only about 30% identical amino acid

TABLE 1. PHB content in mutant strains and wild-type *M. extorquens* AM1 grown on C_1, C_2, and multicarbon substrates

Strain	PHB (% of dry biomass [wt/wt])			
	Methanol	Ethylamine	Pyruvate	Succinate
M. extorquens AM1	22–25	32–30	36–38	17–20
gap11 mutant	9–12	11–13	34–36	30–40
gap20 mutant	3–5	16–18	35–37	4–6
phaR mutant	0–0.2	26–28	1–2	0–1

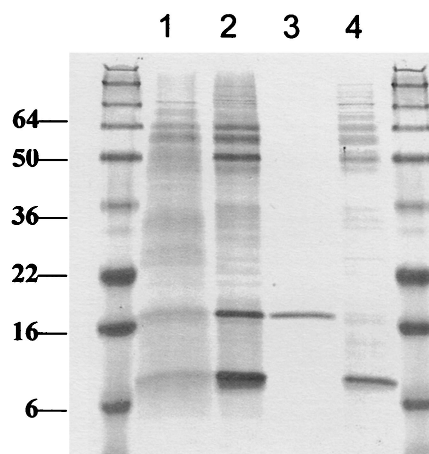

FIG. 2. SDS-PAGE of the proteins associated with PHB granules isolated from wild-type *M. extorquens* AM1 and *gap11* and *gap20* mutants. Lanes 1 and 2, wild type grown on methanol and succinate, respectively; lane 3, *gap11* mutant grown on succinate; lane 4, *gap 20* mutant grown on succinate. Size markers (molecular masses in kilodaltons) are shown in the flanking lanes.

TABLE 2. Activities of catechol dioxygenase (milliunits) in wild-type *M. extorquens* AM1 and the *phaR* mutant carrying transcriptional fusions, grown on succinate or grown on succinate and induced with methanol[a]

Fusion	Wild type		*phaR* mutant	
	Succinate	Methanol	Succinate	Methanol
phaA:xylE	75	135	50	400
phaB:xylE	75	80	50	350
phaC:xylE	175	330	165	700
phaR:xylE	135	385	150	410
gap11:xylE	1,990	1,760	1,885	1,715
gap20:xylE	30	20	25	30

[a] Background activity of XylE expressed without a promoter is about 1 mU.

residues, while identities with the rhizobial AniA polypeptides are much higher (about 56%). In this work, we used a number of approaches to test if PhaR in *M. extorquens* AM1 might be a transcriptional regulator.

We analyzed the amino acid sequence translated from *phaR* for the presence of DNA-binding motifs by using two different programs devoted to helix-turn-helix motif detection (see Materials and Methods). The searches resulted in low scores, predicting that the probability of PhaR being able to bind DNA is low. As controls, we performed the same searches with proteins known to bind DNA, for example, LysR-type regulators from rhizobia, and obtained high scores with the same programs.

To test transcription levels for key genes in PHB biosynthesis, we cloned promoter regions for *phaA*, *phaB*, and *phaC*, encoding the enzymes of PHB biosynthesis, and also promoter regions for *gap11*, *gap20*, and *phaR* upstream of the promoterless *xylE* gene, and tested the activity of XylE in the wild-type background and in the *phaR*-minus background (Table 2). Transcription from *gap11*, *gap20*, and *phaR* promoters was at a similar level in wild-type and *phaR* backgrounds, indicating that *phaR* has no role in transcriptional regulation of phasins in *M. extorquens* AM1, or in its own transcriptional regulation. Transcription from *phaA*, *phaB*, and *phaC* promoters was increased two- to fourfold in the *phaR* background in the methanol-induced cultures, while it was not affected in the succinate-grown cultures. These data were supported by testing the activities of PhaA (β-ketothiolase) and PhaB (acetoacetyl-CoA reductase) in the *phaR* mutant, and they were somewhat increased after methanol induction (PhaA, 0.52 U; PhaB, 0.05U), but not on succinate (PhaA, 0.71 U; PhaB, 0.03U), compared to the wild type (0.46 and 0.03 versus 1.18 and 0.4 U, respectively).

We further tested the ability of PhaR to bind DNA by overexpressing *phaR* in *E. coli* (Fig. 3) and performing gel retardation assays with the resulting cell extracts. All the promoter regions tested for promoter activity and listed in Table 2 were employed. However, no retardation was observed with any of the DNA fragments tested (data not shown). These data suggest that, although transcription of some genes is altered in the *phaR* mutant, PhaR may not act directly as a transcriptional regulator.

Role of PhaR in partitioning the flow of acetyl-CoA. The evidence showing that PhaR is not a transcriptional regulator of phasins suggested that it might play a role more similar to that of AniA. A number of results in the literature point toward AniA, a homolog of PhaR playing a role in directing carbon flow in rhizobial species (12, 36). However, the key metabolite in this regulation or the targets of such a regulation still remain elusive

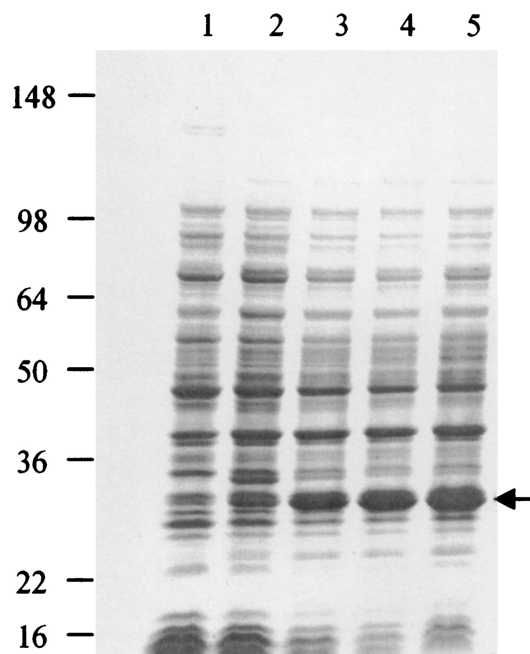

FIG. 3. Expression of PhaR in *E. coli* BL21(DE3). Proteins were separated by SDS-PAGE. Lane 1, total proteins of *E. coli* carrying the plasmid with *phaR* before induction by IPTG; lanes 2 to 5, total cellular proteins of *E. coli* carrying the plasmid with *phaR* after 1, 2, 3, and 4 h of induction by IPTG, respectively. The arrow denotes the PhaR band. Numbers at left are molecular masses in kilodaltons.

In *M. extorquens* AM1, the phenotype of the PhaR mutant points towards PhaR having a role in partitioning the flow of acetyl-CoA into the PHB cycle. Figure 4 shows the different sources and fates of acetyl-CoA in *M. extorquens* AM1 growing on C_1, C_2, and heterotrophic substrates. Based on the schemes shown in Fig. 4 and the presence of significant PhaA and PhaB activity in the *phaR* mutant, the defect in PHB accumulation during growth on C_1 and C_3-C_4 compounds in the *phaR* mutant could be explained if the acetyl-CoA pool is not sufficient for PHB biosynthesis in the absence of PhaR. In that case, the specific growth defect on methanol could be due to low availability of acetyl-CoA for the glyoxylate regeneration cycle, an essential pathway for growth on C_1 compounds (8, 23, 24). The long lag and eventual recovery of growth (Fig. 1A) could reflect the time that it takes to build up an effective acetyl-CoA pool to drive C_1 metabolism. During growth on C_2 compounds, all of the carbon and energy flux passes through acetyl-CoA. This larger, direct flux may bypass the PhaR defect, thereby allowing normal PHB production during growth on C_2 compounds. Several lines of evidence were pursued to test this hypothesis.

A prediction of this hypothesis is that the PhaR mutant should grow normally on methanol in the presence of supplementing acetate. Figure 5 shows that the *phaR* mutant does grow normally on methanol in the presence of 0.1 to 0.2 mM acetate, supporting the idea that the PhaR mutant is defective

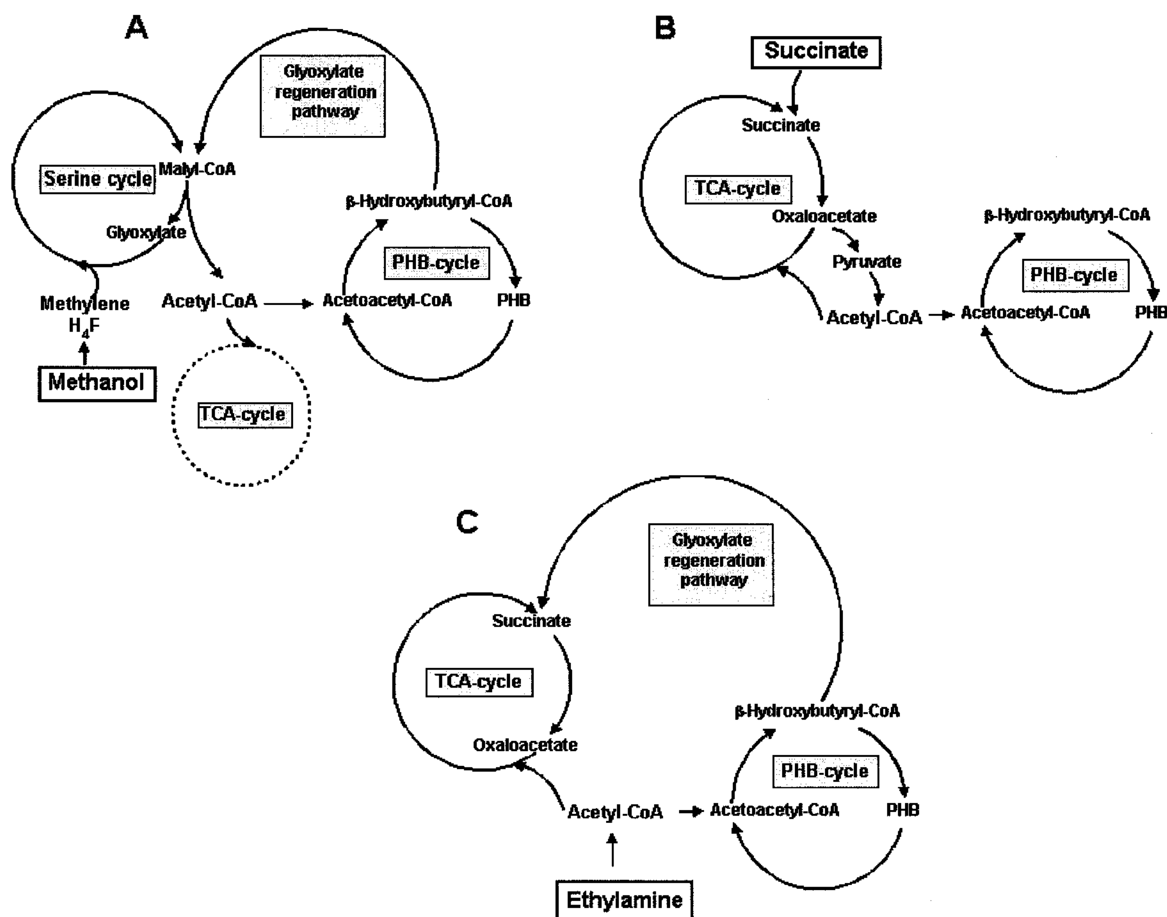

FIG. 4. Schematic representation of major pathways producing and consuming acetyl-CoA in *M. extorquens* AM1 during growth on different substrates. (A) Methanol. The main source of acetyl-CoA is the malyl-CoA lyase reaction (6, 42). The main fate of acetyl-CoA is its conversion to β-hydroxybutyryl-CoA, which is partitioned roughly equally between PHB synthesis and the glyoxylate regeneration pathway (23, 24). Very little acetyl-CoA is oxidized via the TCA cycle during methylotrophic growth. (B) Succinate. The main source of acetyl-CoA is the pyruvate dehydrogenase reaction. The main fate of acetyl-CoA is oxidation through the TCA cycle, with a lesser flux into PHB. (C) Ethylamine. Acetyl-CoA is made directly from the C_2 substrate, and acetyl-CoA is partitioned between the TCA cycle and β-hydroxybutyryl-CoA. A significant flux occurs into both PHB and the glyoxylate regeneration cycle, which generates the C_4 acceptors for acetyl-CoA oxidation via the TCA cycle (23, 24, 41).

in acetyl-CoA production and/or partitioning. *M. extorquens* AM1 does not grow on acetate as a sole source of carbon and energy, and so this growth is not due to utilization of the acetate by itself. We further demonstrated that the addition of higher concentrations of acetate (10 to 50 mM) not only complements growth of the *phaR* mutant on methanol but also restores wild-type levels of PHB production on this substrate, while PHB production on C_3 and C_4 compounds remained unchanged (data not shown).

The partitioning of acetyl-CoA was also tested directly by measuring $^{14}CO_2$ production from 2-^{14}C-labeled acetate in whole cells. As shown in Fig. 4, acetyl-CoA has three major fates in *M. extorquens* AM1, depending on the growth substrate: oxidation by the tricarboxylic acid (TCA) cycle, incorporation into PHB, or conversion to C_4 compounds by the glyoxylate regeneration pathway. It has been shown that the C-1 atom of acetyl-CoA is converted to CO_2 in the glyoxylate regeneration pathway but the C-2 atom is not (24). The C-2 atom, however, is converted to CO_2 in the TCA cycle. Therefore, the production of $^{14}CO_2$ from 2-^{14}C-labeled acetate is a measure of TCA cycle activity. Incorporation of [2-^{14}C]acetate into CO_2 and cells increased linearly with time in *M. extorquens* AM1 and the PhaR mutant during incubation in the presence of methanol (data not shown). Under these conditions, in *M. extorquens* AM1 the proportion of the total [2-^{14}C]acetate utilized that was detected in CO_2 was less than 0.5%, confirming that the TCA cycle functions at very low activity in the wild-type strain during methylotrophic metabolism (42). In the PhaR mutant, the proportion of $^{14}CO_2$ production from the total 2-^{14}C-labeled acetate utilized was about 11%, indicating that in this mutant the TCA cycle activity is greatly increased compared to that in the wild type. These results demonstrate the redistribution of acetyl-CoA partitioning in this mutant, with a significant flux being redirected to the TCA cycle.

DISCUSSION

M. extorquens AM1 accumulates PHB during growth on all substrates tested, and the levels of PHB accumulation seem to

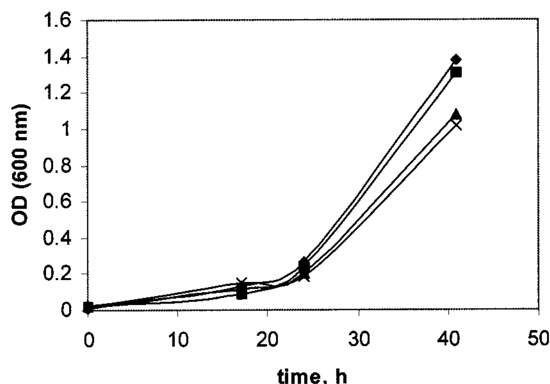

FIG. 5. Growth of *M. extorquens* AM1 and *gap20*, *gap11*, and *phaR* mutants on methanol in the presence of 0.2 mM acetate. Symbols: diamond, *M. extorquens* AM1; square, *gap11* mutant; triangle, *gap20* mutant; cross, *phaR* mutant. OD, optical density.

be determined by the nature of the substrate, suggesting substrate-specific regulatory mechanisms (23). In this work we investigated three genes potentially involved in controlling PHB accumulation in *M. extorquens* AM1, via mutation and phenotypic analysis: *gap11* and *gap20*, encoding GAPs, and *phaR*, encoding a putative regulator.

Phasins have been implicated previously in playing an important role in PHB granule formation, as well as in other aspects of PHB accumulation and degradation (15, 18). Several phasins have been isolated and characterized before, from *R. eutropha* (44), *Rhodococcus ruber* (34, 35), *P. denitrificans* (28), *Chromatium vinosum* (27), and *Pseudomonas oleovorans* (22, 37). In this work we have identified *gap11* and *gap20*, encoding the two phasins in *M. extorquens* AM1. The amino acid sequences translated from *gap 11* and *gap20* reveal low or no identity to phasins characterized from non-*Methylobacterium* strains, while they show a significant degree of identity to each other. Despite the sequence similarity, however, the two phasins differ in size, are expressed at different levels, and apparently fulfill different roles in PHB synthesis regulation, as mutants with mutations in the two phasins have different phenotypes. Further work will be necessary to determine the mechanism of action of these two phasins.

In this study we concentrated on investigating the role of PhaR in *M. extorquens* AM1. While in *P. denitrificans* PhaR was demonstrated elsewhere to be involved in controlling phasin levels as well as its own expression (29), our results indicate that PhaR does not regulate phasins in *M. extorquens* AM1 at the level of transcription. Instead, our evidence suggests that PhaR is involved in controlling metabolite flows, a role suggested elsewhere for its homolog, AniA in rhizobia (12, 36). The mutation in *phaR* virtually eliminates PHB production in *M. extorquens* AM1 during growth on methanol, succinate, and pyruvate and also results in significant growth reduction on methanol. However, a wild-type growth phenotype on methanol and wild-type PHB accumulation on this substrate were restored by the addition of acetate, pointing toward the acetyl-CoA pool as a potential regulation target. This hypothesis is supported by the ability of the *phaR* mutant to grow and synthesize PHB at wild-type levels on ethylamine, when the supply of acetyl-CoA should not be limited. Finally, our results demonstrating that PhaR mutants redirect the flow of acetyl-CoA from PHB precursors to the TCA cycle indicate a role in regulating acetyl-CoA flux. These results may explain the increase in transcription and enzyme activity observed for the PHB-synthesizing enzymes in the PhaR mutant. This increase was initially puzzling, as it is the opposite trend that would be expected to result in low PHB production. However, if PhaR controls acetyl-CoA partitioning in the cell, the metabolic state of the PhaR mutant would be expected to be very different from that of the wild type and could result in significantly altered cellular signals. This in turn could alter regulation of other enzymes in the cell, including the PHB synthesis enzymes, but would not result in increased PHB synthesis if the acetyl-CoA flux was directed elsewhere. In all PHB-synthesizing bacteria the TCA cycle and the PHB cycle compete for acetyl-CoA (9, 26, 32, 33, 39). Most bacteria accumulate PHB only under conditions when carbon is abundant but oxygen, nitrogen, or sulfate is limited (9, 26, 33, 39). Under these conditions cells possess high ratios of NAD(P)H/NAD(P) that are shown to inhibit citrate synthase and isocitrate dehydrogenase, effectively decreasing the rate of acetyl-CoA metabolism via the TCA cycle. Reduced consumption via the TCA cycle redirects the pool of acetyl-CoA into the PHB cycle, with reoxidation of NAD(P)H. In *M. extorquens* AM1, the overlap of the PHB biosynthesis pathway with the central metabolic pathways for C_1 and C_2 assimilation (23, 24) suggests a continuous acetyl-CoA flux via the PHB-synthesizing enzymes β-ketothiolase and acetoacetyl-CoA reductase during C_1 and C_2 growth. In *Methylobacterium rhodesianum*, a methylotrophic bacterium very similar to *M. extorquens* AM1, the affinity of β-ketothiolase (PhaA) for acetyl-CoA was shown previously to be about 20-fold lower than the affinity of citrate synthase (32). However, the flux into the TCA cycle in this bacterium is not significant compared to the flux into both the PHB cycle and the glyoxylate regeneration cycle during growth on methanol. These results imply the presence of specific mechanisms for regulating fluxes of acetyl-CoA in serine cycle methylotrophs, to support the glyoxylate regeneration pathway during methylotrophic growth. Our results show that PhaR is a key player in regulating acetyl-CoA fluxes in *M. extorquens* AM1.

Although the mechanism by which PhaR acts is not yet known, the evidence so far is not supportive of a direct role in transcriptional regulation. It is possible that PhaR regulates either the acetyl-CoA-producing reactions (malyl-CoA lyase and pyruvate dehydrogenase) or the acetyl-CoA-consuming reactions (β-ketothiolase and citrate synthase), perhaps by altering enzyme activity in vivo. Future work to confirm this hypothesis will require experiments with purified enzymes and PhaR. However, the results presented here suggest that PhaR is a central regulator of carbon flow in this serine cycle methylotroph and assists in redirecting carbon through alternative pathways as the cell switches between different metabolic modes.

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