

Connection between Poly- β -Hydroxybutyrate Biosynthesis and Growth on C₁ and C₂ Compounds in the Methylo-troph *Methylobacterium extorquens* AM1

NATALIA KOROTKOVA¹ AND MARY E. LIDSTROM^{1,2*}

Department of Chemical Engineering¹ and Department of Microbiology,² University of Washington, Seattle, Washington 98195-1750

Received 3 August 2000/Accepted 7 November 2000

Several DNA regions containing genes involved in poly- β -hydroxybutyrate (PHB) biosynthesis and degradation and also in fatty acid degradation were identified from genomic sequence data and have been characterized in the serine cycle facultative methylo-troph *Methylobacterium extorquens* AM1. Genes involved in PHB biosynthesis include those encoding β -ketothiolase (*phaA*), NADPH-linked acetoacetyl coenzyme A (acetyl-CoA) reductase (*phaB*), and PHB synthase (*phaC*). *phaA* and *phaB* are closely linked on the chromosome together with a third gene with identity to a regulator of PHB granule-associated protein, referred to as *orf3*. *phaC* was unlinked to *phaA* and *phaB*. Genes involved in PHB degradation include two unlinked genes predicted to encode intracellular PHB depolymerases (*depA* and *depB*). These genes show a high level of identity with each other at both DNA and amino acid levels. In addition, a gene encoding β -hydroxybutyrate dehydrogenase (*hbd*) was identified. Insertion mutations were introduced into *depA*, *depB*, *phaA*, *phaB*, *phaC*, and *hbd* and also in a gene predicted to encode crotonase (*croA*), which is involved in fatty acid degradation, to investigate their role in PHB cycling. Mutants in *depA*, *depB*, *hbd*, and *croA* all produced normal levels of PHB, and the only growth phenotype observed was the inability of the *hbd* mutant to grow on β -hydroxybutyrate. However, the *phaA*, *phaB*, and *phaC* mutants all showed defects in PHB synthesis. Surprisingly, these mutants also showed defects in growth on C₁ and C₂ compounds and, for *phaB*, these defects were rescued by glyoxylate supplementation. These results suggest that β -hydroxybutyryl-CoA is an intermediate in the unknown pathway that converts acetyl-CoA to glyoxylate in methylo-trophs and *Streptomyces* spp.

Many prokaryotes, including aerobic methylo-trophic bacteria, accumulate poly- β -hydroxybutyrate (PHB) intracellularly as a carbon and energy reserve material. Methylo-trophic bacteria employing the serine cycle for formaldehyde assimilation are able to accumulate up to 80% of PHB by dry weight, while methylo-trophic bacteria with the Calvin-Benson-Bassham cycle of C₁ assimilation can synthesize up to 20% of PHB by dry weight (14). In obligate methanol utiliz-ers, which use the ribulose monophosphate pathway for assimilation of reduced C₁ compounds, PHB has not been detected (14).

Two pathways for PHB synthesis are known in bacteria. In *Ralstonia eutropha*, *Methylobacterium extorquens*, *Zoogloea ramigera*, and *Azotobacter beijerinckii* PHB is synthesized from acetyl coenzyme A (acetyl-CoA) as a result of sequential action of three enzymes: β -ketothiolase, NADPH-linked acetoacetyl-CoA reductase, and PHB synthase (5, 15, 19, 33, 34) (Fig. 1). In *Rhodospirillum rubrum* and *Methylobacterium rhodezianum* PHB synthesis is catalyzed by five enzymes: β -ketothiolase, NADH-linked acetoacetyl-CoA reductase, L-(+) and D-(-)-specific crotonyl-CoA hydratases (crotonases), and PHB synthase (27, 28, 29).

The degradation of PHB in most bacteria is catalyzed by PHB depolymerase, β -hydroxybutyrate dehydrogenase, acetoacetate-succinate-CoA transferase and β -ketothiolase (Fig. 1).

β -Ketothiolase, NADH-linked acetoacetyl-CoA reductase, L-crotonase, and crotonyl-CoA reductase are also involved in the fatty acid degradation pathway (1) (Fig. 1).

Genes responsible for polyhydroxyalkanoic acid (PHA) synthesis have been isolated and characterized from several bacteria. In *R. eutropha* the genes encoding β -ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*), and PHB synthase (*phaC*) are closely linked on the chromosome (33, 38, 42). In *Rhizobium meliloti*, *Paracoccus denitrificans*, and *Z. ramigera* the two genes, *phaA* and *phaB*, are organized in an operon (32, 44, 48). In *P. denitrificans* *phaC* is located on the chromosome together with genes encoding PHB depolymerase (*orf1*), PHB granule-associated protein (*phaP*), and a regulator for PHB granule-associated protein (*phaR*) (23). The PHB synthase gene (*phaC*) was isolated and characterized from *M. extorquens* strain IBT no.6. In this strain, it has been shown that *phaC* is located separately from other genes encoding enzymes for PHB biosynthesis (45).

In order to understand carbon flow during methylo-trophic growth of *M. extorquens* AM1, it is necessary to understand PHB synthesis and degradation, since cells growing exponentially on methanol accumulate a significant proportion of their biomass as PHB (42). The goal of the current study was to characterize genes participating in PHB cycling in *M. extorquens* AM1 and to determine their roles in methylo-trophic metabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5 α (Bethesda Research Laboratories) and S17-1 (40) were used in the study. They were grown in the Luria-Bertani medium in the presence of appropriate antibi-

* Corresponding author. Mailing address: Department of Chemical Engineering, University of Washington, Box 351750, Seattle, WA 98195-1750. (206) 616-5282. Fax: (206) 616-5721. E-mail: lidstrom@u.washington.edu.

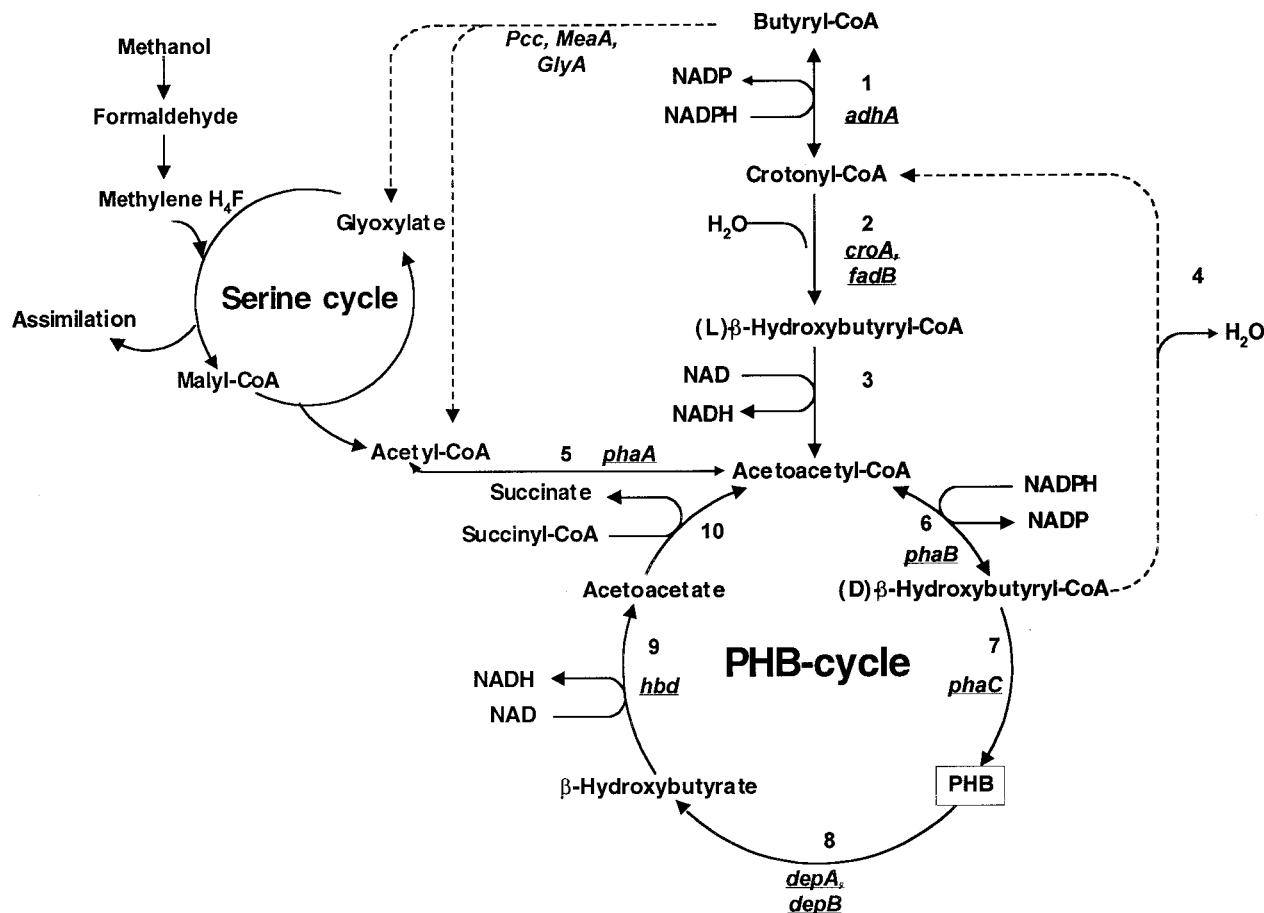


FIG. 1. Pathways of PHB metabolism and fatty acid degradation in *M. extorquens* AM1 and their connection to methylotrophy. Enzymes of the fatty acid degradation pathway: 1, crotonyl-CoA reductase; 2, L-crotonase; 3, NADH-linked acetoacetyl-CoA reductase; 4, D-crotonase. Enzymes of PHB synthesis and degradation: 5, β -ketothiolase; 6, NADPH-linked acetoacetyl-CoA reductase; 7, PHB synthase; 8, PHB depolymerase; 9, β -hydroxybutyrate dehydrogenase; 10, acetoacetate-succinyl-CoA transferase. Dotted lines reflect enzymes not yet known or detected.

otics as described by Maniatis et al. (24). *M. extorquens* AM1 was grown in a minimal medium described previously (18). Succinate (20 mM), methanol (100 mM), ethanol (50 mM), ethylamine (20 mM), formate (40 mM), pyruvate (40 mM), or β -hydroxybutyrate (20 mM) were used as substrates. The following antibiotic concentrations were used for *M. extorquens* AM1: tetracycline, 10 $\mu\text{g ml}^{-1}$; kanamycin, 100 $\mu\text{g ml}^{-1}$; and rifamycin, 50 $\mu\text{g ml}^{-1}$. The growth responses of mutants were tested on plates containing the substrates listed above in the presence or absence of supplements of glyoxylate (5 mM), glycolate (20 mM), acetoacetate (0.1%), β -hydroxybutyrate (0.1%), or butyrate (0.04%). The following cloning vectors were used: pUC19 (Pharmacia) for cloning and subcloning, pAYC61 (8) as a suicide vector, pRK2013 (12) as a helper plasmid, and pCR2.1 (Invitrogen) for cloning of PCR products. In addition, a recently developed small IncP plasmid, pCM80 (C. Marx and M. Lidstrom, unpublished data), was used. pCM80 is an expression vector designed for use in *M. extorquens* AM1 that utilizes the strong promoter upstream of the methanol dehydrogenase gene cluster (26).

Cloning of PHB cycle genes. Data from the *M. extorquens* AM1 genome project (<http://faculty.washington.edu/lidstrom/genome.html>) were used for designing primers specific for putative genes of *M. extorquens* AM1 encoding the following enzymes: intracellular PHB depolymerases (phb1, 5'-TCGCCGACCGCCGTG AAC-3', and phb2 reverse, 5'-GCGAGATACTCGTCGTAGAAG-3', complementary to nucleotides 608 to 625 and nucleotides 861 to 841, respectively, with respect to the translation start site of gene encoding first depolymerase; phb3, 5'-GTGATGTCGTCCTTCTCGCC-3', and phb4 reverse 5'-GCTTGATTCAA GCGAGCTGC-3', complementary to nucleotides 1024 to 1005 and nucleotides -38 to -19, respectively, with respect to the translation start site of gene encoding second depolymerase), acetoacetyl-CoA reductase (re1, 5'-GAACGC GTCGCCCTCGTCACG-3', and re2, 5'-GCCGTTGATCGACGAGATG-3',

complementary to nucleotides 10 to 30 and nucleotides 416 to 398, respectively, with respect to the translation start site of gene encoding acetoacetyl-CoA reductase), β -ketothiolase (th1, 5'-CGCACTGGAGCGCGCAGGCG-3', and th2, 5'-CGAAAGCCTCGTTCGCCTCG-3', complementary to nucleotides 116 to 135 and nucleotides 960 to 941, respectively, with respect to the translation start site of gene encoding β -ketothiolase), β -hydroxybutyrate dehydrogenase (hbd1, 5'-CATCGCCAAGCACTTCGCC-3', and hbd2, 5'-CCGGTGATCTGGC TGGCGTG-3', complementary to nucleotides 59 to 77 and nucleotides 745 to 725, respectively, with respect to the translation start site of gene encoding β -hydroxybutyrate dehydrogenase), and crotonase (kr1, 5'-CATATAGGTCAT TACGATCAG-3', and kr2, 5'-GCCTGAGACGCTTGACGAGGAAC-3', complementary to nucleotides 755 to 736 and nucleotides -29 to -7, respectively, with respect to the translation start of gene encoding crotonase). GenBank data (<http://www.ncbi.nlm.nih.gov>) were used for the design of primers specific to the gene encoding PHA synthase of *M. extorquens* AM1. The primers used were as follows: s1, 5'-GCAACGATCTGATGGAGTTG-3', and s2, 5'-GATCCTCGA CGATCAGCCAG-3', which is complementary to nucleotides 721 to 740 and nucleotides 2417 to 2398, respectively, with respect to the translation start of gene encoding PHA synthase.

Cloning of transhydrogenase genes. A 3.05-kb PCR fragment containing *pntA* and *pntB* of *E. coli* (GenBank accession no. AAC7467) was cloned into pCR2.1, the resulting plasmid was digested with *HindIII-XbaI*, and this fragment was cloned into pCM80 in the correct orientation with respect to the *mxvF* promoter. The resulting plasmid was transferred into *phaA* and *phaB* mutants of *M. extorquens* AM1 by conjugation.

Cloning of isocitrate lyase gene. A 1.31-kb PCR product containing *icl* of *E. coli* (GenBank accession no. X12431) was cloned into pCR2.1, restricted by *HindIII-XbaI*, and cloned into pCM80 in the correct orientation with respect to

the *mxrF* and *lacZ* promoters. The resulting plasmid was transferred into *phaA* and *phaB* mutants of *M. extorquens* AM1.

DNA-DNA hybridization. Preparation of nitrocellulose filters and DNA probes for DNA-DNA hybridizations was carried out as described previously (16).

DNA manipulations. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, or filling in ends with Klenow enzyme were carried out as described by Maniatis et al. (24). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure of Saito and Miura (36).

DNA sequencing. DNA sequencing from both strands was carried out with an Applied Biosystems automated sequencer by the Department of Biochemistry, University of Washington Sequencing Facility.

Computer analysis. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using Genetics Computer Group and NCBI ORF Finder programs (<http://www.ncbi.nlm.nih.gov>).

Enzyme assays. Enzyme activities were determined 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. Pyridine nucleotide transhydrogenase was assayed as described by Zahl et al. (49). β -Ketothiolase (EC 2.3.1.16), acetoacetyl-CoA reductase (EC 1.1.1.36), and β -hydroxybutyrate dehydrogenase were determined by the methods of Senior and Dawes (39). L-(+)- and D-(-)-specific enoyl-CoA hydratases (EC 4.2.1.17) were measured as described by Shuto et al. (40). Isocitrate lyase was assayed according to the method of Copeland et al. (11). Spectrophotometric methods (21, 47) were used for protein determination.

Matings. Triparental or biparental matings between *E. coli* 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 (12) was used as a helper plasmid. Rifamycin was used for *E. coli* counterselection.

PHB analysis. The PHB concentration in bacterial cells was determined by a gas chromatographic method (6). A model GC-14A capillary gas chromatograph (Shimadzu, Kyoto, Japan) with an AT-WAX capillary column (0.53 mm by 10 m; 1.2- μ m film thickness) (Alltech, Deerfield, Ill.) and a flame ionization detector were 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.

Nucleotide sequence accession number. The sequences of 1,206 nucleotides containing *depA* and of 6,100 nucleotides containing *phaA* and *phaB* have been deposited with GenBank under accession numbers AF287908 and AF287907, respectively.

RESULTS

Nucleotide sequence analysis. Putative genes for the two intracellular PHB depolymerases, acetoacetyl-CoA reductase, β -ketothiolase, β -hydroxybutyrate dehydrogenase, crotonase, and PHA synthase were identified in the course of an ongoing *M. extorquens* AM1 genome sequencing project, by performing automated BLAST searches on raw single-read sequences from the *M. extorquens* AM1 genomic database (<http://faculty.washington.edu/lidstrom/genome.html>) by the high level of identity of the translated polypeptide sequences to, respectively, the intracellular PHB depolymerase of *R. eutropha*, the β -ketothiolase and acetoacetyl-CoA reductase of *Z. ramigera*, the β -hydroxybutyrate dehydrogenase of *S. meliloti*, the crotonase of *Thermoanaerobacterium thermosaccharolyticum*, and the PHA synthase of *M. extorquens* (3, 25, 32, 45). Primers were designed complementary to the coding regions of these sequences and used to amplify seven corresponding fragments by PCR. After the identities of the PCR products were confirmed by sequencing, three PCR fragments encoding PHB depolymerase, acetoacetyl-CoA reductase, and β -ketothiolase were used as probes against a cosmid library of *M. extorquens* AM1 chromosomal DNA (16). Two types of cosmids were identified by DNA-DNA (Southern) hybridization in filters: one that hybridized with both β -ketothiolase and acetoacetyl-CoA re-

ductase probes and one that hybridized with the probe for PHB depolymerase. *Hind*III fragments from selected cosmids that hybridized to the appropriate probes were cloned into pUC19 and partially sequenced.

The complete PHB depolymerase gene was sequenced and designated *depA* (Fig. 2, top line). The amino acid sequence translated from *depA* showed the highest level of identity (47%) with the intracellular PHB depolymerase from *R. eutropha* (GenBank accession no. BAA33394). The two surrounding open reading frames (ORFs) showed identities (54 and 29%, respectively) with a putative histidine kinase from *Bradyrhizobium japonicum* (GenBank accession no. CAA06858) and a hypothetical protein from *Deinococcus radiodurans* (GenBank accession no. AAF12030). These were designated *regS* and *orf1*, respectively (Fig. 2).

The physical map of the DNA region containing genes for β -ketothiolase and acetoacetyl-CoA reductase is shown in Fig. 2 in the center line. The polypeptide translated from the first ORF showed the highest level of identity (60%) with the ribosomal protein L32 of *Neisseria meningitidis* (GenBank accession no. CAB83837 [31]). This gene was designated *orf2*. The polypeptide translated from the second ORF showed the highest identities with an unknown protein located in the *phb* locus of *S. meliloti* (GenBank accession no. AAA90981; 52% identity [3]) and also with a regulator for granule-associated protein (*phaR*) in *P. denitrificans* (GenBank accession no. BAA77259; 32% identity [23]) and *phbF* of *R. eutropha* (GenBank accession no. AAC3819; 41% identity [42]). This gene was designated *orf3*. The third ORF encodes a polypeptide that is similar to β -ketothiolase (PhaA) of *Z. ramigera* (GenBank accession no. 7766963; 67% identity [25]). This gene was designated *phaA*. The next ORF located downstream from *phaA* and its translated product showed a high degree of identity with acetoacetyl-CoA reductase (PhaB) from *Z. ramigera* (GenBank accession no. P23238; 67% identity [32]). This gene was designated *phaB*. The polypeptides translated from the two last ORFs showed identities with lauroyl acyltransferase (*htrB*) of *Rickettsia prowazekii* (GenBank accession no. CAA15149; 26% identity [2]) and aminopeptidase of *Thermus aquaticus* (GenBank accession no. P42778; 57% identity [30]), respectively. These genes were designated *orf4* and *orf5*.

The remaining genes of interest were completely or partially sequenced from the corresponding PCR fragments. The complete gene for the second PHB depolymerase (*depB*) was identified within the 1,060-bp PCR fragment (Fig. 2, bottom line). The polypeptide translated from *depB* showed high identity with the intracellular PHB depolymerase of *R. eutropha* (GenBank accession no. BAA33394; 49% identity) and *depA* of *M. extorquens* AM1 described above (72% identity). The gene for β -hydroxybutyrate dehydrogenase (*hbd*) was identified as a partial ORF within the 700-bp PCR fragment. The translated product of *hbd* showed high identity with β -hydroxybutyrate dehydrogenase (Hbd) of *S. meliloti* (GenBank accession no. AAD12733; 60% identity in the 229-amino-acid overlap [3]). The gene for crotonase (*croA*) was identified as a partial ORF in the 791-bp PCR product. The product translated from *croA* showed identity with crotonase (CroA) of *T. thermosaccharolyticum* (GenBank accession no. CAB07495; 36% identity in a 164-amino-acid overlap). The last PCR fragment contained 1,700 bp encoding two partial ORFs. The partial ORFs showed

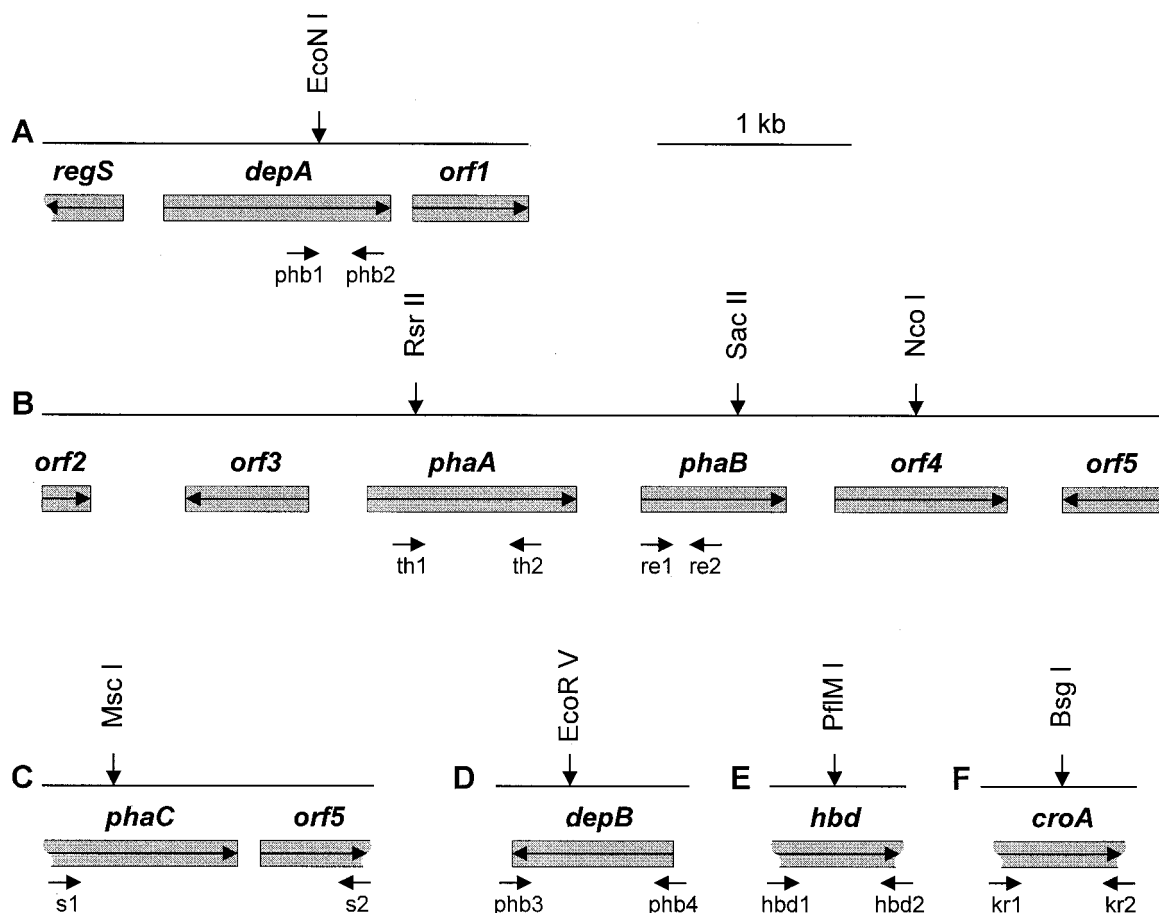


FIG. 2. Physical map of the *M. extorquens* AM1 chromosomal regions analyzed in this study. Only restriction sites discussed here are shown. Arrows within the boxes indicate the direction of transcription, vertical arrows indicate the sites of insertion mutations, and the small arrows underneath indicate the primers used to generate PCR products.

100% identity at the nucleotide sequence level to polyhydroxyalkanoic acid synthase (PhaC) from *M. extorquens* strain IBT no.6 and a hypothetical protein in the *phaC* 3' region of the same strain (GenBank accession no. AAA72330 [45]), respectively. These genes were designated *phaC* and *orf6*, respectively (Fig. 2).

Construction of insertion mutations. Insertion mutations in *depA*, *depB*, *orf4*, *phaA*, *phaB*, *phaC*, *hbd*, and *croA* were constructed in vitro using the kanamycin resistance (Km^r) gene cartridge as described earlier (8). The mutation sites for each gene are indicated in Fig. 2. Mutants were selected 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 with primers specific to the insertion sites.

Phenotypic analysis of insertion mutants. (i) Growth substrates. Mutations in all the genes produced double-crossover recombinants (null mutants) on succinate plates with a frequency of 10 to 50% relative to the total number of recombinants. These results demonstrated that none of these genes were required for growth on succinate. Growth responses were determined for double-crossover recombinant insertion mutants. All mutants grew on both succinate and pyruvate on

plates, and all grew normally on a combination of methanol and succinate, demonstrating that none were methanol sensitive. In addition, *depA*, *depB*, and *croA* mutants grew normally on all compounds tested, including C_1 compounds (methanol and formate), C_2 compounds (ethylamine and ethanol), and β -hydroxybutyrate. The mutant in *hbd* grew normally on methanol, formate, ethylamine, and ethanol but did not grow on β -hydroxybutyrate. During growth on methanol, this mutant accumulated β -hydroxybutyrate (Fig. 3).

In contrast to the other mutants, the *phaA*, *phaB*, and *phaC* mutants showed multiple growth defects (Table 1). *phaA* and *phaB* mutants grew fairly well on plates on succinate. However, in liquid they both grew more slowly on succinate than wild-type *M. extorquens* AM1, with a longer growth lag and about half the growth rate of the wild type (Fig. 4). The *phaA* mutant also was unable to grow on methanol, formate, ethylamine, ethanol, or β -hydroxybutyrate, indicating that the product of *phaA* is required for both C_1 and C_2 metabolism. The *phaB* mutant grew poorly on methanol and formate compared to wild-type *M. extorquens* AM1 and did not grow on ethylamine, ethanol, or β -hydroxybutyrate (Table 1). The *phaC* mutant grew more slowly on methanol, ethylamine, ethanol, and β -hydroxybutyrate than wild-type *M. extorquens* AM1. However, this mutant produced with high frequency second-site suppres-

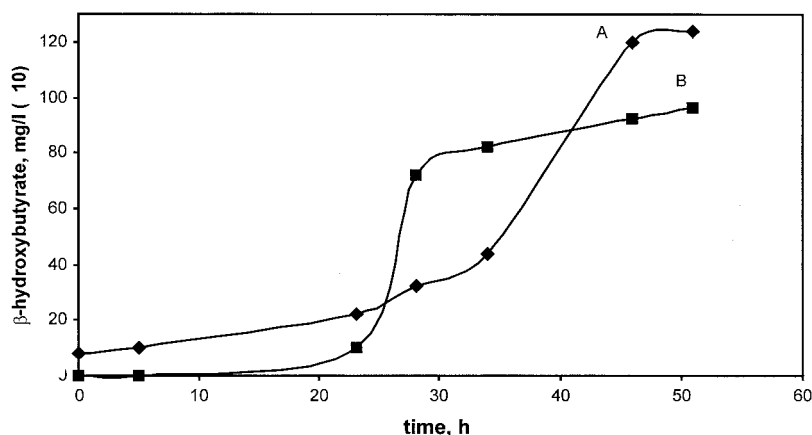


FIG. 3. Characteristics of cell growth (A) and 3-hydroxybutyrate accumulation (B) of the *hbd* mutant grown on methanol. Cell growth is measured by optical density at 600 nm (values shown on y axis have been multiplied by 100).

mutants on methanol and ethylamine media. These grew normally on methanol, ethylamine, ethanol, and β -hydroxybutyrate, but were not revertants, as they still contained the Km insertion in the correct site.

The simultaneous loss of ability to grow on C₁ and C₂ compounds is associated with defects in an unknown portion of the serine cycle for formaldehyde assimilation that functions to oxidize acetyl-CoA to glyoxylate (10, 13). Therefore, the phenotypes of the *phaA* and *phaB* mutants suggested that these genes might be involved in this unknown acetyl-CoA oxidation pathway. Mutants defective in this pathway are characterized by the ability to grow on C₁ and C₂ compounds when supplemented with glyoxylate or glycolate (13). These compounds were tested for the ability to restore the growth of the *phaA* and *phaB* mutants on methanol, ethylamine, and ethanol. Both supplements were able to restore growth of the *phaB* mutant on C₁ and C₂ compounds but did not affect the growth of the *phaA* mutant. However, β -hydroxybutyrate or acetoacetate were able to partially rescue growth of the *phaA* mutant on methanol (Fig. 5). It was not possible to test the growth of the *phaC* mutants in liquid culture due to the outgrowth of the suppressor mutants.

(ii) **Enzyme activities.** The activities of β -ketothiolase and NADPH- and NADH-linked acetoacetyl-CoA reductase were measured in wild type and in *phaA* and *phaB* mutants grown on succinate (Table 2). β -Ketothiolase activity was not detectable in the *phaA* mutant, while it was present in wild-type *M. extorquens* AM1 and the *phaB* mutant, confirming that *phaA* is necessary for β -ketothiolase activity. NADPH-linked aceto-

acetyl-CoA reductase activity was not found in the *phaB* mutant, while it was present at a low level in wild-type *M. extorquens* AM1 and the *phaA* mutant. Low levels of NADH-linked acetoacetyl-CoA reductase were found in the *phaA* and *phaB* mutants and in wild-type *M. extorquens* AM1. These results show that *phaB* is necessary for NADPH-linked acetoacetyl-CoA reductase and that a separate enzyme must be present that accounts for NADH-linked acetoacetyl-CoA reductase activity.

Further proof of the identity of *phaB* was obtained by over-expressing this gene in *E. coli* and *M. extorquens* AM1. A 1.39-kb PCR product containing *phaB* was cloned into pCR2.1 and subsequently subcloned into pCM80 in the correct orientation with respect to the *mxoF* and *lacZ* promoters. The plasmid was transferred into *M. extorquens* AM1. The activity of acetoacetyl-CoA reductase with NADH and NADPH were measured in *E. coli* and *M. extorquens* AM1 containing *phaB* and also in plasmid-free strains. In *E. coli* carrying *phaB*, high NADPH-linked acetoacetyl-CoA reductase activity (71 mU/mg) was found, while no activity was detected in the plasmid-free strain. The NADH-linked activity was at the same low level (about 1 mU/mg) in the control and plasmid-bearing strains. In *M. extorquens* AM1 containing the construct with *phaB*, the activity of the NADPH-linked acetoacetyl-CoA reductase increased to 42 mU/mg of protein in *M. extorquens* AM1. These results identify *phaB* as the gene encoding the NADPH-specific acetoacetyl-CoA reductase.

The L-(+)- and D-(-)-specific crotonase activities were measured in wild-type *M. extorquens* AM1 and in the *croA* mutant

TABLE 1. Growth responses of *pha* mutants on plates

Mutant	Growth response ^a on:									
	Succinate	Pyruvate	Methanol plus succinate	Methanol	Ethanol	Ethylamine	Methanol plus glyoxylate or glycolate	Ethylamine plus glyoxylate or glycolate	Formate	β -Hydroxybutyrate
<i>phaA</i>	++	++	++	-	-	-	-	-	-	-
<i>phaB</i>	++	++	++	+	-	-	++	++	+	-
<i>phaC</i> ^b	++	++	++	+	+	+	NT	NT	+	+

^a ++, Similar to wild type; +, decrease compared to wild type; -, no growth; NT, not tested.

^b Generates second site suppressor mutants at high frequency.

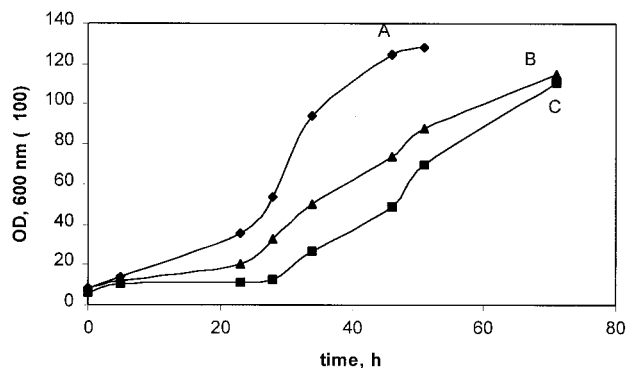


FIG. 4. Growth of *M. extorquens* AM1 (A) and the *phaA* (C) and *phaB* (B) mutants on succinate.

grown on succinate or methanol. D(-)-Specific crotonase was not detectable in wild-type *M. extorquens* AM1 or the mutant. L(+)-specific crotonase was present in both wild-type *M. extorquens* AM1 and the *croA* mutant (Table 2). These data suggested that *M. extorquens* AM1 may have multiple genes responsible for crotonase activity. This idea is supported by the fact that a putative gene for the α -subunit of the fatty oxidation complex (which includes enoyl-CoA hydratase, β -hydroxyacyl-CoA dehydrogenase, and β -hydroxybutyryl-CoA epimerase) was also identified in the course of our *M. extorquens* AM1 genome sequencing project. The gene product showed identity (44% in a 231-amino-acid overlap) with the translated polypeptide sequence of *fadB* (putative fatty oxidation protein) of *Mycobacterium tuberculosis* (GenBank accession no. CAA17666).

The activity of NAD-linked β -hydroxybutyrate dehydrogenase was measured in wild-type *M. extorquens* AM1 and the *hbd* mutant grown on succinate. The activity was not detectable in the *hbd* mutant, while it was present in wild-type *M. extorquens* AM1 at a low level (Table 2), confirming the necessity of *hbd* for β -hydroxybutyrate dehydrogenase activity.

(iii) **PHB accumulation.** PHB concentrations were determined as the percentage of dry weight in *depA*, *depB*, *phaA*, *phaB*, *phaC*, *hbd*, and *croA* mutants and also in wild-type *M. extorquens* AM1 grown on succinate or methanol (Table 3).

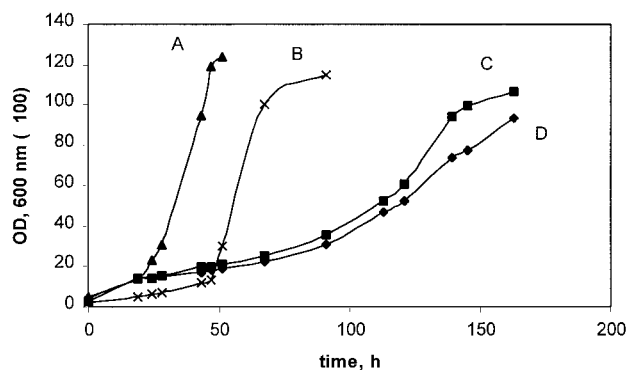


FIG. 5. Growth of *M. extorquens* AM1 on methanol (A) and on methanol in the presence of 0.1% β -hydroxybutyrate (B) and of *phaA* mutant on methanol in the presence of 0.1% acetoacetate (C) and in the presence of 0.1% β -hydroxybutyrate (D).

depA, *depB*, *hbd*, and *croA* mutants accumulated normal levels of PHB (15 to 20% on succinate and 30 to 43% on methanol). *phaA*, *phaB*, and *phaC* mutants did not accumulate detectable PHB during growth on succinate. The *phaB* mutant accumulated a small amount (7 to 10%) of PHB during growth on methanol, demonstrating the presence of an alternate pathway for β -hydroxybutyryl-CoA synthesis. It is possible that this pathway uses enzymes participating in fatty acid degradation (Fig. 1). The second site suppressor *phaC* mutants showing wild-type growth characteristics (see above) also did not accumulate PHB during growth on methanol or ethylamine, confirming that they retained the PHB synthesis defect of the original *phaC* mutant.

Expression of *E. coli* pyridine nucleotide transhydrogenase in *phaA* and *phaB* mutants. It has been suggested that PHB can serve as a redox regulator, maintaining NADPH balance in the cell (1). It seemed possible that the *pha* defects might result in an increase in NADPH pools, which could in turn cause inhibition of key NADPH-dependent enzymes important for C_1 and C_2 metabolism and might cause the growth defects. To test this hypothesis, the *pntA* and *pntB* genes of *E. coli*, encoding the α and β subunits of pyridine nucleotide transhydrogenase were expressed in *phaA* and *phaB* mutants. Transhydrogenase expression should result in decreased NADPH levels in the mutants. The activity of pyridine nucleotide transhydrogenase was measured in *phaA* and *phaB* mutants carrying *pntA* and *pntB* genes transcribed from the *mxoF* promoter and shown to be present at higher levels (63 and 92 mU/mg in *phaA* and *phaB* mutants containing the construct with *pntA* and *pntB*, respectively) than in the mutants without the construct (13 mU/mg in *phaA* and *phaB* mutants). However, these transconjugants did not regain the ability to grow on methanol or ethylamine plates. These results show that while a significant increase in transhydrogenase activity was achieved, it had no effect on the growth of the mutants.

Expression of *icl* of *E. coli* in *phaA* and *phaB* mutants. To verify the participation of *phaA* and *phaB* in the oxidation of acetyl-CoA to glyoxylate, the isocitrate lyase gene (*icl*) of *E. coli* was expressed in *phaA* and *phaB* mutants. Isocitrate lyase should allow the completion of the glyoxylate cycle in *M. extorquens* AM1 and should bypass the need for the native acetyl-CoA oxidation pathway. In *phaA* and *phaB* mutants carrying *icl*, the activity of isocitrate lyase was detected (89 and 242 mU/mg, respectively), while no activity was found in the plasmid-free mutants. The *phaA* and *phaB* transconjugants did not grow on ethylamine. However, the *phaA* transconjugants grew very slowly on methanol (Fig. 6). The *phaB* transconjugants grew more slowly on methanol than wild-type *M. extorquens* AM1 (Fig. 6). These data suggest that the NADPH-linked acetoacetyl-CoA reductase and β -ketothiolase are involved in the unknown pathway for glyoxylate regeneration in *M. extorquens* AM1.

DISCUSSION

In this study, we have used preliminary genome sequence data to assess genes in *M. extorquens* AM1 involved in the biosynthesis and degradation of PHB and also in fatty acid degradation. Synthesis of PHB as a reserve material is a common feature among methylobacteria with the serine pathway for C_1 assimilation (14). *M. extorquens* has been reported to pro-

TABLE 2. Activity of PHB cycling enzymes in extracts of succinate-grown *M. extorquens* AM1 wild-type and mutants.

Enzyme	Activity (nmol/min/mg of protein) ^a				
	AM1	PhaA ⁻	PhaB ⁻	Cro ⁻	Hbd ⁻
β-Ketothiolase	392	0	400	NT	NT
Acetoacetyl-CoA reductase					
NADH-dependent	17	12	11	NT	NT
NADPH-dependent	13	10	0	NT	NT
L-(+)-Crotonase	25	NT	NT	17	NT
NAD-dependent β-hydroxybutyrate dehydrogenase	9	NT	NT	NT	0

^a Values are the average of three to four determinations; the range was ±10%. AM1, wild-type *M. extorquens* AM1; 0, not detectable; NT, not tested.

duce PHB at up to 64% of the dry cell mass (43). Three enzymes are involved in PHB synthesis in *M. extorquens*: β-ketothiolase, NADPH-linked acetoacetyl-CoA reductase, and PHB synthase. Degradation of PHB in *M. extorquens* is catalyzed by PHB depolymerase, β-hydroxybutyrate dehydrogenase, acetoacetate-succinate-CoA transferase, and β-ketothiolase.

We have shown that the genes encoding β-ketothiolase and NADPH-linked acetoacetyl-CoA reductase (*phaA* and *phaB*) are closely linked on the *M. extorquens* AM1 chromosome and are transcribed in the same direction. It is not yet known whether the two are cotranscribed. Another gene was found in the region whose translated product showed identity with the regulator for a PHB granule-associated protein of *P. denitrificans*. In addition, *M. extorquens* AM1 has two genes, *depA* and *depB*, predicted to encode intracellular PHB depolymerase. These genes show high identity to each other and are located on different chromosomal fragments.

Mutants unable to synthesize PHB have been isolated from several bacterial species, i.e., *R. eutropha*, *R. rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sphaerodes*, *Rhizobium etli*, and *R. meliloti* (7, 20, 22, 35, 37). Most mutants are defective in the PHB synthase gene (the *phaC* equivalent), and in most cases no obvious growth phenotype is associated with the inability to synthesize PHB. However, in *R. etli*, a *phaC* mutant showed reduced growth characteristics on minimal medium in comparison with wild-type strains and excreted large amounts of organic acids (7). In *R. capsulatus* a *phaC* mutant was sensitive to fatty acids (22). However, *R. capsulatus phaA* and *phaAB* mutants grew normally on tested substrates and were able to synthesize PHB, suggesting the presence of an alternative route for β-hydroxybutyryl-CoA synthesis (22).

In contrast to the results in *R. capsulatus* the *phaA* mutant of

M. extorquens AM1 did not accumulate PHB, while the *phaB* mutant accumulated reduced amounts of PHB on methanol but did not accumulate PHB on succinate. These results indicate that both *phaA* and *phaB* are central to PHB biosynthesis in *M. extorquens* AM1. It is likely that a low-level alternate route of β-hydroxybutyryl-CoA synthesis is present that accounts for the small amount of PHB in methanol-grown cells.

Surprisingly, *M. extorquens* AM1 *phaA*, *phaB*, and *phaC* mutants showed growth defects on both C₁ and C₂ compounds. The defects are most severe in the *phaA* mutant, but the *phaB* mutant also exhibited very poor growth on C₁ compounds and no growth on C₂ compounds. The *phaC* mutant could grow on all tested substrates but at a slower rate on C₁ and C₂ compounds. Moreover, the *phaB* mutant phenotype on methanol and ethylamine was rescued by the addition of glyoxylate or glycolate, while the *phaA* mutant phenotype on methanol was partially rescued by the addition of β-hydroxybutyrate or acetoacetate.

A possible explanation for the growth phenotypes of the mutants is that the lack of PHB synthesis causes NADPH or NADH to accumulate to inhibitory levels by preventing NADPH consumption. NADH accumulation has been demonstrated in *phaC* mutants in *R. etli*, and inhibition of tricarboxylic acid cycle enzymes by NADH has been proposed as an explanation for the slow growth and organic acid excretion by this mutant when grown on glucose and succinate (7). Our results suggest this explanation is unlikely for *M. extorquens* AM1 because the mutants show growth defects on formate. Formate is a low-energy growth substrate and the only direct source of reducing equivalents in cells grown on formate is NADH produced via formate dehydrogenase. Therefore,

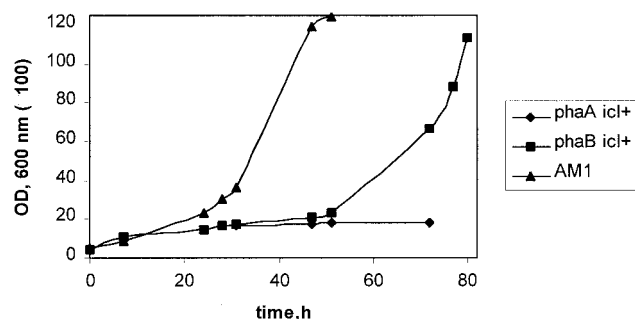


FIG. 6. Growth on methanol of *M. extorquens* AM1 wild type and of the *phaA* and *phaB* transconjugants containing the *E. coli* *icl*.

TABLE 3. Biosynthesis of PHB by *M. extorquens* AM1 and mutants during growth on methanol and succinate

Strain	Carbon source	PHB (% [dry biomass wt]) ^a
<i>M. extorquens</i> AM1	Succinate	18–20
<i>M. extorquens</i> AM1	Methanol	34–42
Mutants ^b	Succinate	15–20
Mutants ^b	Methanol	30–43
<i>phaA</i> mutant	Succinate	0 ²
<i>phaB</i> mutant	Succinate	0
<i>phaB</i> mutant	Methanol	7–9
<i>phaC</i> mutant	Succinate	0
<i>phaC</i> suppressor mutant	Methanol	0

^a Range of three determinations. 0, Not detectable.

^b One *depA* mutant, one *depB* mutant, one *croA* mutant, and one *hbd* mutant.

NADPH should be limiting in this growth condition and NADH should not be in excess. Another line of evidence in this regard is the result from overexpression of pyridine nucleotide transhydrogenase. *M. extorquens* AM1 shows a low activity of transhydrogenase, and the main sink for NADPH in methanol- and ethylamine-grown cells is PHB synthesis. Therefore, it is possible that NADPH accumulates in the PHB⁻ mutants, which might become inhibitory on these growth substrates. However, overexpression of the pyridine nucleotide transhydrogenase in these mutants did not affect their phenotypes. If NADPH accumulation were the main reason for the growth defect, then we might expect that some alleviation would occur under these conditions. In addition, it is not clear why glyoxylate or glycolate would rescue the growth phenotype of *phaB* mutants if the accumulation of reduced pyridine nucleotides were a major reason for the defects.

The alternative possibility is that β -ketothiolase and NADPH-linked acetoacetyl-CoA reductase are involved in the unknown pathway for glyoxylate regeneration from acetyl-CoA, since other mutants defective in this pathway show growth on C₁ and C₂ compounds in the presence of glyoxylate or glycolate. Serine cycle methylotrophs such as *M. extorquens* AM1 lack isocitrate lyase, and the pathway used for assimilation of acetate, which also operates during growth on C₁ compounds and β -hydroxybutyrate, remains unknown. It has been shown previously using experiments with ¹⁴C-labeled C₂ compounds that glyoxylate and glycolate are intermediates in this pathway (13).

Previously two *M. extorquens* AM1 chromosomal fragments have been identified as carrying genes encoding enzymes involved in assimilation of both C₁ and C₂ compounds and probably operating in the unknown pathway for glyoxylate regeneration. One of these, *glyA*, encodes serine hydroxymethyltransferase. This enzyme probably plays a dual role in *M. extorquens* AM1: both as the first enzyme in the serine cycle and as an enzyme in the glyoxylate regeneration pathway (9). The other chromosomal fragment is not linked to the fragment mentioned above and has been shown to contain three genes involved in the unknown pathway for glyoxylate regeneration, i.e., *meaA*, *pccA*, and *adhA* (10). The *pccA* gene product has been demonstrated to be a propionyl-CoA carboxylase (10). *adhA* has strong similarity to *ccr*, the gene encoding a NADPH-linked crotonyl-CoA reductase in *Streptomyces collinus* (17). This enzyme catalyzes the conversion of crotonyl-CoA to butyryl-CoA. We have recently shown that *adhA* of *M. extorquens* AM1 does encode the NADP-linked crotonyl-CoA reductase (enzyme 1 in Fig. 1; L. V. Chistoserdova and M. E. Lidstrom, unpublished results).

meaA encodes a novel CoB₁₂-dependent mutase that is not methylmalonyl-CoA mutase or isobutyryl-CoA mutase (10, 17). A similar gene is present downstream of *ccr* in *S. collinus*. *meaA*- and *ccr*-blocked mutants of *S. collinus* exhibit dramatically reduced growth characteristics when acetate is the sole carbon source (17). This result suggests that both *ccr* and *meaA* are probably involved in the unknown pathway of acetate assimilation in *S. collinus*, similar to the one operating in *M. extorquens* AM1.

The phenotypes of the new mutants of *M. extorquens* AM1 described here indicate that the initial step of the glyoxylate regeneration pathway may be proceeding via intermediates of

the PHB biosynthesis pathway (enzymes 5 and 6 in Fig. 1) and reversible reactions of fatty acid degradation (enzymes 1 and 4 in Fig. 1). It is not clear why the *phaA* mutants are not rescued for growth on C₁ and C₂ compounds by glyoxylate or glycolate. It is possible that these mutants accumulate an intermediate that has an inhibitory action or that they have low levels of an intermediate that is required for enzyme activation or for other pathways. These mutants are partially rescued for growth on C₁ and C₂ compounds by acetoacetate and β -hydroxybutyrate, pointing to acetoacetate or acetoacetyl-CoA as possible key intermediates in the unknown pathway.

As noted above, the *M. extorquens* AM1 *phaB* mutant accumulates low concentrations of PHB during growth on methanol. This phenotype indicates that an alternative mechanism for β -hydroxybutyryl-CoA production must be present in *M. extorquens* AM1. It is likely that NADH-linked acetoacetyl-CoA reductase (enzyme 3 in Fig. 1) and L- and D-crotonases (enzymes 2 and 4 in Fig. 1) supply PHB biosynthesis with a β -hydroxybutyryl-CoA pool and account for the slow growth of the mutants on methanol. Although we were unable to detect D-crotonase activity in cell extracts, it is possible that the activity is labile or requires special conditions to detect. Alternatively, another unknown pathway for D- β -hydroxybutyryl-CoA synthesis may exist in *M. extorquens* AM1.

The *phaC* mutant grows slowly on methanol and ethylamine but forms second site suppressor mutants on these substrates with high frequency. These suppressor mutants are not able to accumulate PHB, but they do regain the ability to grow normally on methanol or ethylamine, demonstrating that the growth defects are not due to the inability to synthesize PHB per se. These results suggest that PHB synthase is not required for assimilation of C₁ and C₂ compounds. Although more detailed studies will be necessary to determine the nature of the suppressors, it is possible that the *phaC* mutants accumulate an inhibitory intermediate or block the production of another key intermediate, as with the *phaA* mutants. In this case, the suppressor mutants may upregulate or downregulate a consumption or production pathway or alter a target of the inhibition. Since suppressor mutants do not accumulate at a significant frequency in the *phaA* mutants, the phenotypes must be due to different effects.

The mutant in *hbd* has lost the ability to use β -hydroxybutyrate as sole carbon source confirming participation of *hbd* in the first step of β -hydroxybutyrate utilization. In addition, these mutants accumulate β -hydroxybutyrate without affecting growth on methanol, indicating that this intermediate is not inhibitory at these levels. The *croA*, *depA*, *depB*, and *orf4* mutants had a wild-type phenotype. Thus, we have shown that β -hydroxybutyrate dehydrogenase and the *croA*, *depA*, *depB*, and *orf4* products are not required for the assimilation of C₁ and C₂ compounds.

The details of the pathway for converting acetyl-CoA to glyoxylate in *M. extorquens* AM1 and other bacteria lacking isocitrate lyase are still not known. However, the results presented here strongly suggest that the first part of this pathway involves the conversion of two acetyl-CoA molecules to butyryl-CoA (Fig. 1).

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