Requirement for the Enzymes Acetoacetyl Coenzyme A Synthetase and Poly-3-Hydroxybutyrate (PHB) Synthase for Growth of Sinorhizobium meliloti on PHB Cycle Intermediates

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We have identified two Sinorhizobium meliloti chromosomal loci affecting the poly-3-hydroxybutyrate degradation pathway. One locus was identified as the gene acsA, encoding acetoacetyl coenzyme A (acetoacetyl-CoA) synthetase. Analysis of the acsA nucleotide sequence revealed that this gene encodes a putative protein with a molecular weight of 72,000 that shows similarity to acetyl-CoA synthetase in other organisms. Acetyl-CoA synthetase activity was not affected in cell extracts of glucose-grown acsA Tn5 mutants; instead, acetoacetyl-CoA synthetase activity was drastically reduced. These findings suggest that acetoacetyl-CoA synthetase, rather than CoA transferase, activates acetoacetate to acetoacetyl-CoA in the Sinorhizobium meliloti poly-3-hydroxybutyrate cycle. The second locus was identified as phbC, encoding poly-3-hydroxybutyrate synthase, and was found to be required for synthesis of poly-3-hydroxybutyrate deposits.

The catabolism of intracellular carbon stores is a strategy employed by many bacterial species to survive nutritionally suboptimal conditions. Polyhydroxylalkanoates (PHA), such as poly-3-hydroxybutyrate (PHB), accumulate in cells when growth is limited but carbon availability is not (2). This stored carbon can then be utilized during conditions of otherwise limiting carbon availability (45). PHB synthesis and degradation are collectively referred to as the PHB cycle. The extent of PHB accumulation is dependent on the relative rates of synthesis and degradation, which in turn are controlled by growth conditions (36, 41).

PHA have attracted substantial industrial interest for their use as high-quality, biodegradable plastics (2). This interest has driven research efforts directed at PHA biosynthesis. The genes encoding the enzymes responsible for PHA synthesis have been isolated and characterized from a number of bacterial species (33, 43), including Sinorhizobium meliloti strains Rm41 (49) and Rm1021 (51). The degradative portion of the cycle has not been subjected to similar attention, and it is less well defined both genetically and biochemically.

We have recently isolated a number of S. meliloti mutants that are affected in the ability to utilize PHB cycle intermediates as sole carbon sources to support growth (11). The mutations mapped to loci on both the chromosome and the pRmeSU47b (pEXO) megaplasmid. Two loci on the megaplasmid have been identified via enzymatic and nucleotide sequence analyses. One encodes the enzyme 3-hydroxybutyrate dehydrogenase (3), and the other encodes methylmalonyl coenzyme A (methylmalonyl-CoA) mutase (10). In this paper, we further characterize and identify two of the chromosomal loci.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. The construction of new strains is described in the text. Bacterial culture in Luria-Bertani (LB) and TY complex media and modified M9 conditions (36, 41).

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TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td>Sinorhizobium meliloti</td>
<td></td>
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</tr>
<tr>
<td>Rm1021</td>
<td>SU47 str-2f (Sm&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>Rm5320</td>
<td>Rm1021 pRmeSU47αf30::Tn5-11 (mobilizable pRmeSU47a)</td>
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<tr>
<td>Rm11105</td>
<td>pbbC::Tn5 (aac1-1::Tn5)</td>
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<td>Rm11134</td>
<td>aclA7::Tn5 (aac1-7::Tn5)</td>
<td>11</td>
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<td>Rm11135</td>
<td>aclA8::Tn5 (aac1-8::Tn5)</td>
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<td>Rm11144</td>
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<tr>
<td>Rm11149</td>
<td>aclA49::Tns5-B20</td>
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<td>Rm11153</td>
<td>pbbC::Tn5 (aac1-1::Tn5)</td>
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<td>Rm11160</td>
<td>aclA7::Tn5-233 (aac1-7::Tn5-233)</td>
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<td>Rm11368</td>
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<td>DH5a</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; endA1 hisdR17 (rK&lt;sub&gt;11&lt;/sub&gt; m&lt;sub&gt;1&lt;/sub&gt; K) supE44 thi1 recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 d800lacZΔM15, λ&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>MT607</td>
<td>pro-82 thi1 hisdR17 supE44 recA56</td>
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<tr>
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<td>MT0670(Tn5)</td>
<td>T. Finan, unpublished</td>
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<tr>
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<td>MT607(Tn5-B20)</td>
<td>17</td>
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<td>CS8 R&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt;, cured of pTC58 and pATC58</td>
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<td>pSP329</td>
<td>IncP cloning vector, Te&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>pVK101</td>
<td>IncP cloning vector, Km&lt;sup&gt;+&lt;/sup&gt; Te&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<td>Cloning vector, CoIE1 oriV, bla</td>
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</tr>
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<td>pTC338</td>
<td>PLAFR1 clone, Te&lt;sup&gt;+&lt;/sup&gt; complements aau&lt;sup&gt;-1&lt;/sup&gt; mutant</td>
<td>This study</td>
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<tr>
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<td>7-kb EcoRI fragment from pTC338 in pUC18, contains aclA</td>
<td>This study</td>
</tr>
<tr>
<td>pTC355</td>
<td>7-kb EcoRI fragment from pTC338 in pVK101, contains aclA</td>
<td>This study</td>
</tr>
<tr>
<td>pTC381</td>
<td>aau&lt;sup&gt;-1&lt;/sup&gt;:Tn'&lt;sup&gt;+&lt;/sup&gt; EcoRI self-ligation product, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pGQ105</td>
<td>aau&lt;sup&gt;-1&lt;/sup&gt; and aau&lt;sup&gt;-7&lt;/sup&gt; complementing 4-kb KpnI fragment from pTC350 in pSP329, Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pGQ109</td>
<td>HindIII fragment from pTC338 in pUC18</td>
<td>This study</td>
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* Abbreviations for antibiotics: Km, kanamycin; Rf, rifampin; Sm, streptomycin; Te, tetracycline.

RESULTS

Isolation of complementing clones. The pLAFR1 cosmid clone bank was introduced into the mutant strains Rm11105 and Rm11134, harboring mutations in the aau<sup>-1</sup> and aau<sup>-7</sup> loci, respectively. Both of these mutations render strains carrying them unable to utilize the PHB cycle intermediates 3-hydroxybutyrate and acetocacetate as sole carbon sources. Following selection on M9 supplemented with 3-hydroxybutyrate as the sole carbon source, we obtained two cosmid clones, designated pTC338 (from the Rm11105 complementation) and pGQ2 (from the Rm11134 complementation). Complementation was confirmed by reintroduction of the clones into the mutant strains by conjugation. Surprisingly, both pTC338 and pGQ2 complemented each of the two mutants. Restriction analysis confirmed that these two clones shared common-sized EcoRI fragments (data not shown). A common 7-kb EcoRI fragment was subcloned from pTC338 into the unique EcoRI site of pVK101. The resulting plasmid, pTC355, was able to complement both mutants. Further subcloning experiments determined that a 4-kb KpnI subfragment of the 7-kb EcoRI fragment (pGQ105) was also capable of complementation.

Since aau<sup>-1</sup> and aau<sup>-7</sup> were previously mapped to two distinct regions on the S. meliloti chromosome (11), the complementation could not be homologous in the case of both loci. At least one of the two loci must have been unlinked to the 7-kb EcoRI fragment. To resolve this, a Tn5-B20 insertion which abolished the ability to complement strain Rm11105 for 3-hydroxybutyrate utilization was isolated in pTC355. This inser-
tion was then recombined into the genome by homogenization, resulting in strain Rm11149. Transduction mapping indicated the insertion to be 100% (60 of 60) linked to the aau-7::Tn5-233 insertion in strain Rm11160 and unlinked (none of 50) to the aau-1::Tn5-233 insertion in strain Rm11144. Southern blot analysis (data not presented) confirmed that the aau-7::Tn5 insertion was located in the 7-kb EcoRI fragment. Therefore, pTC355 appears to reverse the aau-7 phenotype by homologous complementation and the aau-1 phenotype by nonhomologous complementation.

Genetic and sequence analysis of aau-7 complementing region. To better define the region of cosmid clone pGQ2 responsible for complementation of the aau-7 phenotype, 14 independent Tn5 insertions which abolished aau-7 complementation ability were isolated in that clone. All of these insertions were located in the 4-kb KpnI fragment. EcoRI fragments, containing the Tn5 insertions, were subcloned from each of the 14 pGQ2::Tn5 plasmids into pUC18. Subclones of each of the two possible orientations for each insertion were retained. One side of each subclone was deleted by cleavage with HindIII followed by self-ligation, and the sequence of the DNA flanking each insertion was obtained using the ISS0 primer. In this way, the precise site of each Tn5 insertion was determined. Combined with the sequence obtained using custom-designed primers, the sequence assembled into a bidirectional contig of 3,295 bp, extending to the distal KpnI site. Analysis of the sequence revealed a single open reading frame (ORF) of 1,950 bp (650 amino acids) encoding a predicted gene product with a molecular weight of 72,000 (Fig. 1). The (ORF) of 1,950 bp (650 amino acids) encoding a predicted gene product with a molecular weight of 72,000 (Fig. 1). The gene product, which was in turn replaced by Tn5, was first replaced with aau-1::Tn5-233 (Gm' Sp'), which was in turn replaced by Tn5 to make strain Rm11153. Tn5 contains the replication origin derived from pSC101 and is devoid of EcoRI sites (23). By self-ligation of EcoRI-digested genomic DNA from strain Rm11153, followed by transformation of E. coli DH5α to Km', a clone consisting of Tn5 flanked by the aau-1 EcoRI fragment was isolated and designated pTC381. A fragment containing the Tn5-flanking regions of pTC381, along with the ends of the ISS0 elements, was released by HindIII digestion and subcloned into HindIII-digested pUC18. One side of the flanking region was deleted by cleavage with EcoRI followed by self-ligation, and the sequence of the Tn5 flanking region was obtained using the ISS0 primer. The sequence (185 bp) is identical to the segment of the phbc gene from 2532 to 2717 in S. meliloti 1021 (51) and exhibits 98% (181/185) identity to the corresponding segment of the phbc gene in S. meliloti 41 (49).

Since a homologously complementing clone of aau-1 was not obtained, we decided to identify the exact site of insertion of the aau-1::Tn5 insertion. The aau-1::Tn5 was first replaced with aau-1::Tn5-233 (Gm' Sp'), which was in turn replaced by Tn5 to make strain Rm11153. Tn5 contains the replication origin derived from pSC101 and is devoid of EcoRI sites (23). By self-ligation of EcoRI-digested genomic DNA from strain Rm11153, followed by transformation of E. coli DH5α to Km', a clone consisting of Tn5 flanked by the aau-1 EcoRI fragment was isolated and designated pTC381. A fragment containing the Tn5-flanking regions of pTC381, along with the ends of the ISS0 elements, was released by HindIII digestion and subcloned into HindIII-digested pUC18. One side of the flanking region was deleted by cleavage with EcoRI followed by self-ligation, and the sequence of the Tn5 flanking region was obtained using the ISS0 primer. The sequence (185 bp) is identical to the segment of the phbc gene from 2532 to 2717 in S. meliloti 1021 (51) and exhibits 98% (181/185) identity to the corresponding segment of the phbc gene in S. meliloti 41 (49).

We had previously reported the chromosomal location of aau-1 (11), while phbc was more recently reported to map to the megaplasmid pRmeSU47a (51). To resolve the incongruity between the two reports, we attempted to mobilize aau-1::Tn5 by using the pRmeSU47a-located Tn5-11 insertion (≈Tn5 containing oriT, Gm' Sm') in strain Rm5320. The Tn5-11 insertion from several organisms (15, 18, 24, 25, 30, 39, 50) (Table 2), although the other sequences are more similar to each other than they are to the S. meliloti sequence.

To determine the exact site of insertion of the aau-7::Tn5 insertion in strain Rm11134, this mutation was transferred by recombination from the S. meliloti genome onto plasmid pTC338, which carries DNA homologous to the insertion site. First, plasmid pTC338 was introduced into strain Rm11134. Next, pTC338 was conjugated into E. coli MT607, and transconjugants were selected for growth on LB-Km-Tc at 37°C after 24 h. The Km' EcoRI fragment was subcloned into pUC18, BamHI deletions were generated, and sequence was obtained using the ISS0 primer. The site of insertion is defined by a 9-bp repeat of nucleotides 2056 to 2065 (Fig. 1), corresponding to an interruption of sequence at amino acid residue 503.

Identification of aau-1. Since a homologously complementing clone of aau-1 was not obtained, we decided to identify the exact site of insertion of the aau-1::Tn5 insertion. The aau-1::Tn5 was first replaced with aau-1::Tn5-233 (Gm' Sp'), which was in turn replaced by Tn5 to make strain Rm11153. Tn5 contains the replication origin derived from pSC101 and is devoid of EcoRI sites (23). By self-ligation of EcoRI-digested genomic DNA from strain Rm11153, followed by transformation of E. coli DH5α to Km', a clone consisting of Tn5 flanked by the aau-1 EcoRI fragment was isolated and designated pTC381. A fragment containing the Tn5-flanking regions of pTC381, along with the ends of the ISS0 elements, was released by HindIII digestion and subcloned into HindIII-digested pUC18. One side of the flanking region was deleted by cleavage with EcoRI followed by self-ligation, and the sequence of the Tn5 flanking region was obtained using the ISS0 primer. The sequence (185 bp) is identical to the segment of the phbc gene from 2532 to 2717 in S. meliloti 1021 (51) and exhibits 98% (181/185) identity to the corresponding segment of the phbc gene in S. meliloti 41 (49).

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FIG. 1. Physical map of the 7-kb EcoRI fragment and the 3.295-bp sequenced region containing S. meliloti acsA. The hatched bar indicates the ORF of 1,950 bp. The horizontal arrow indicates the direction of transcription. The vertical arrow shows the site of aau-7::Tn5 (=aau-7::Tn5) insertion.

FIG. 2. Promoter region of S. meliloti acsA. The putative ribosome binding site is underlined and labeled “S/D”; the putative promoter is underlined and indicated by “−10” and “−35”.

The putative promoter motif 24 bp upstream. There are also several σ70-type promoter motifs (47) in the upstream region (Fig. 2), although σ24 (gpoN) mutants are not defective in growth on 3-hydroxybutyrate (data not presented). The G+C content of 64.4% for this ORF is comparable to the average G+C content of 61.6% in the S. meliloti genome, and the codon usage is similar to the preference in S. meliloti (data not shown). No ORF longer than 25 amino acid residues was observed between the end of this ORF and the distal KpnI site. Clustal W (46) analysis shows that the 650-amino-acid sequence exhibits homology to acetyl-CoA synthetase sequences.
TABLE 2. Amino acid identities and similarities between acetoacetyl-CoA synthetase and acetyl-CoA synthetasesa

<table>
<thead>
<tr>
<th>Source</th>
<th>% Identity (% similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.m.</td>
<td>24 (40) —</td>
</tr>
<tr>
<td>M.s.</td>
<td>24 (42) 43 (59) —</td>
</tr>
<tr>
<td>P.b.</td>
<td>24 (39) 41 (59) 56 (74) —</td>
</tr>
<tr>
<td>S.c.-2</td>
<td>25 (41) 46 (62) 47 (62) 43 (59) —</td>
</tr>
<tr>
<td>R.e.</td>
<td>23 (39) 43 (59) 50 (66) 45 (61) 50 (66) —</td>
</tr>
<tr>
<td>E.c.</td>
<td>22 (38) 37 (56) 55 (70) 55 (70) 46 (62) 45 (63) —</td>
</tr>
<tr>
<td>B.s.</td>
<td>17 (31) 33 (46) 31 (45) 30 (46) 31 (47) 32 (46) 27 (41) —</td>
</tr>
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</table>

a GenBank accession numbers for the analyzed sequences: S.m. (Sinorhizobium meliloti), AFF08217; M.s. (Methanothrix soehngenii), P27095; P.b. (Phymyces blakesleeanus), S46276; B.s. (Bacillus subtilis), P39062; S.c. (Saccharomyces cerevisiae), S30019; S.c.-2 (S. cerevisiae acs2), P25210; R.e. (Ralstonia eutropha), P31683; E.c. (Escherichia coli), P27550.

b Values were derived using MacVector 6.0.1 (Clustal W algorithm). —, not applicable.

in strain Rm5320 was therefore transduced into strain Rm11105, and the resulting transductant was designated Rm11368. Rm11368 was mated with Agrobacterium strain GM9023, followed by selection for transconjugants on TY-Rf-Gm-Sp and TY-Rf-Nm. Transconjugants arose only on TY-Rf-Gm-Sp, and 50 of these transconjugant colonies were screened for Nmr; 6 of these transconjugant colonies were screened for Nmr; all were found to be Nmr. This result clearly excludes a pRMesSU47a location for phbC and is consistent with our earlier chromosome mapping data (11) and recently reported genomic sequence data (9).

Biochemical characterization of the mutants. A series of enzyme assays was carried out with cell extracts of representative mutants (Table 3). The level of acetyl-CoA synthetase was not reduced in any mutant strain, including the aau-7 mutant. In an attempt to rationalize the aau-7 mutant phenotype with biochemical function, acetoacetyl-CoA synthetase activity was assayed and found to be drastically reduced in the aau-7 mutant strain. In addition, homologous complementation of the aau-7 mutant strain with plasmid pGQ105 restored acetoacetyl-CoA synthetase activity and actually increased it to a level greater than four times higher than the wild-type level. This confirms that aau-7 encodes acetoacetyl-CoA synthetase (acetoacetyl-CoA ligase; EC 6.2.1.16), which activates acetoacetate to acetoacetyl-CoA by the single reaction ATP + CoA + acetoacetate =\rightarrow acetoacetyl-CoA + AMP + PP. We have thus designated the gene acsA. Although the aau-7 strain exhibited lower than wild-type succinyl-CoA transferase activity, the presence of acsA-bearing pGQ105 did not increase the activity to a level significantly greater than that in the wild-type strain. Although the aau-1 mutant exhibited slightly reduced levels of acetoacetyl-CoA synthetase activity and ketohiolase activities, this perhaps reflects physiological effects related to reduced provision of acetoacetate substrate in the absence of accumulated PHB. The slightly reduced levels of 3-ketothiolase activities in each of the other mutant strains perhaps reflect lower levels of available acetoacetyl-CoA substrate in these backgrounds. The aau-1 mutant did not accumulate PHB after growth in YM, while all other strains accumulated PHB to 60 to 70% of cell dry mass, thus further confirming the synonymity of aau-1 and phbC, encoding PHB synthase.

Physiological traits. To determine whether the ability to accumulate or metabolize PHB deposits affects cell survival ability, we designed a carbon starvation assay to investigate the starvation survival of the PHB-negative phbC mutants and the PHB degradation-deficient acsA mutant. Strains were cultivated under PHB-accumulating conditions in YM to stationary phase, transferred to carbon nutrient-free M9 medium, and incubated. Viable counting after the transfer to carbon nutrient-free medium indicates that the mutant strains do not propagate as well as the wild-type strain (Fig. 3) upon initial subculture. It is presumably because the wild-type strain is utilizing the accumulated PHB stores, while the mutant strains cannot store or utilize PHB. After 1 month of incubation, however, none of the cultures had dropped below the initial titer.

The symbiotic properties of the mutants were investigated by inoculation of axenic alfalfa seedlings cultivated in the absence of fixed nitrogen. All mutants formed root nodules which fixed N₂, as evidenced by the green shoots and shoot dry weight similar to wild type (data not shown).

DISCUSSION

We have demonstrated that acsA, encoding acetoacetyl-CoA synthetase, is required for acetoacetate metabolism in S. meliloti. Unlike the acetyl-CoA synthetase enzymes of other organisms which are rarely able to use C₄ fatty acids as substrates (28, 37, 39), it appears that acetoacetate is the substrate for the S. meliloti AcS. The absence of any significant ORFs downstream of acsA on the smallest complementing subclone indicates that the carbon utilization phenotype is not caused by polar effects on a downstream gene. This work establishes that in S. meliloti, acetoacetyl-CoA synthetase is responsible for activation of acetoacetate, even in the presence of considerable levels of acetoacetate:succinyl-CoA transferase activity. Therefore, we propose that acetoacetyl-CoA synthetase activity is an integral part of the degradation portion of the PHB cycle in S. meliloti. To our knowledge, this is the first report of the mutation and sequence determination of a gene encoding acetoacetyl-CoA synthetase.

Three distinct mechanisms for activation of acetoacetate to acetoacetyl-CoA have been found in bacteria. In E. coli, acetoacetate is activated by a CoA transferase (encoded by adoD and adoA) and then converted to two molecules of acetyl-CoA by ketothiolase (encoded by adoB) (27). It has been suggested that in other bacteria, such as Azotobacter beijerinckii, acetoacetate is activated by an acetoacetate:succinyl-CoA transfer-
mid-encoded (multiple-copy) acsA to suppress the phbC growth phenotype and is also consistent with our recent observation that disruption of phbB, encoding acetacetyl-CoA reductase, also affects growth on acetocetate (P. Anjea et al., unpublished data).

The demonstration that symbiotic N₂ fixation ability is not affected in any of the PHB cycle mutant strains is consistent with reports indicating that neither PHB synthesis nor degradation is required for effective symbiosis (3, 38, 51), although PHB synthesis has been shown to be important for symbiotic competence (51). PHB is accumulated as an endogenous source of carbon and energy, and fluorescence microscopy provided visual evidence of PHB utilization during carbon-free starvation in Legionella pneumophila (26). This is consistent with our finding that during carbon-free starvation of S. meliloti, PHB synthesis and PHB degradation mutants showed reduced ability to proliferate during the first 30 days of incubation. The mutants were not, however, deficient in the ability to survive prolonged cultivation in the absence of an external nutrient carbon source.

**ACKNOWLEDGMENTS**

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