Poly-β-hydroxybutyrate (PHB) biosynthetic genes in *Rhizobium meliloti* 41

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**INTRODUCTION**

In the last two decades much research has been done on polyesters synthesized and accumulated by microorganisms belonging to diverse taxonomic groups (Anderson & Dawes, 1990; Steinbüchel, 1991). Natural polymers exist in which tens of hydroxyalkanoic acids have been detected. The chemical family that groups all these polymers is that of the polyhydroxyalkanoic acids (PHA). In this paper we refer to PHB as a PHA whose monomers are hydroxybutyric acid and/or hydroxyvaleric acid. The thermoplastic properties of some of these polymers have given rise to great scientific and industrial interest. *Rhizobium* species accumulate PHB as reserve material (Forsyth et al., 1958; Tombolini & Nuti, 1989). In the bacterial cells this polymer functions as a carbon storage compound or as a sink for reducing equivalents. *Rhizobium* has not yet been thoroughly investigated as a potential PHA producer for industrial purposes. It has been suggested that PHB in *Rhizobium* bacteroids may play an important role in nitrogen fixation efficiency (Wong & Evans, 1971; Kretovich et al., 1977; Romanov et al., 1980; Bergersen et al., 1991; Bergersen & Turner, 1992, 1993). Controlled utilization of PHB in bacteroids may sustain N₂-fixation under suboptimal plant carbon supply conditions (Bergersen & Turner, 1992). The genetics of PHA production has been in part elucidated; the *Alcaligenes eutrophus* operon containing *phaC* (PHA synthase), *phaA* (β-ketothiolase, EC 2.3.1.16) and *phaB* (NADPH-dependent acetoacetyl-CoA reductase, EC 1.1.1.36) when expressed in *Escherichia coli* enables the formation of PHB granules indistinguishable from those found in the original host (Schubert et al., 1988; Slater et al., 1988; Peoples & Sinskey, 1989a, b). In addition, the following genes have been cloned and sequenced from other bacteria: *phaA, B, C* from *Chromatium vinosum* (Liebergessl & Steinbüchel, 1992) and *Acinetobacter* sp. (Schembri et al., 1994a, b); *phaA, C* from *Thiocystis violacea* (Liebergessl & Steinbüchel, 1993); *phaC* from *Rhodobacter sphaeroides* (Hustede et al., 1992; Hustede & Steinbüchel, 1993), *Methyllobacterium extorquens* (Valentin & Steinbüchel, 1993) and *Rhodococcus ruber* (Pieper & Steinbüchel, 1992); and *phaA, B* from *Zoogloeas ramigera* (Peoples et al., 1987; Peoples & Sinskey, 1989c). In some instances, a fourth gene, *phaE*, has been demonstrated to be required for PHA synthesis. It was found in *Chromatium vinosum* and *Thiocystis violacea* and is involved in the polymerization step of PHA biosynthesis (Liebergessl & Steinbüchel, 1992, 1993; Liebergessl et al., 1992). Recently the product of an ORF in the PHA synthase gene locus of *Rhodococcus ruber* was identified as a protein present on the granule surface (Pieper-Fürst et al., 1994). The finding of PHA leaky mutants that map in genes not directly involved in PHB biosynthesis (Fries et al., 1991, 1992) and the

**Keywords**: *Rhizobium meliloti*, poly-β-hydroxybutyrate, *phaA, phaB, phaC*
presence of ORFs structurally linked to *phaA*, *phaB* and *phaC* (Steinbüchel et al., 1992) indicate the possible involvement of additional genes. In a previous report (Povolo et al., 1994) we described the isolation and analysis of *Rhizobium meliloti* PHB mutants carrying a Tn5 in the synthase gene. The present study reports the cloning and characterization of the genes involved in PHB biosynthesis in this species.

**METHODS**

**Bacterial strains and plasmids.** *Rhizobium meliloti* and *Escherichia coli* strains, and plasmids, used in this study are listed in Table 1.

**Growth conditions.** *R. meliloti* was grown at 30°C in YMB medium (per litre: 0.5 g K2HPO4, 0.2 g MgSO4, 7H2O, 0.1 g NaCl, 10.0 g mannitol, 0.4 g yeast extract; pH 6.8), TY medium (per litre: 5.0 g tryptone, 3.0 g yeast extract, 0.5 g KH2PO4, 0.5 g MgSO4, 7H2O, 0.15 g NaCl, 0.01 g CaCl2, 0.5 g NH4NO3, 2.0 g sucrose, 2.5 mg FeSO4, 7H2O, 0.5 mg MnSO4·H2O, 0.5 mg ZnSO4·7H2O, 0.5 mg CuSO4·5H2O, 0.5 mg H3BO3, 0.5 mg NaMoO4, 0.01 mg thiamin, 0.01 mg calcium-pantothenate, 0.01 mg thiamin). When required, antibiotics were added at the following concentrations: 50 µg kanamycin ml–1, 100 µg rifampicin ml–1. *E. coli* was grown at 37°C in LB medium (Sambrook et al., 1989), supplemented with antibiotics as appropriate (20 µg ampicillin ml–1, 30 µg kanamycin ml–1, 10 µg tetracycline ml–1). Media were solidified by the addition of 1·5 % (w/v) agar. Liquid cultures were stirred at 100 r.p.m.

**DNA isolation and manipulation.** Plasmid DNA was prepared from crude lysates by alkaline extraction (Sambrook et al., 1989). DNA fragments were isolated from agarose gels using a gel extraction kit (Qiagen). Cosmid DNA was digested with restriction endonucleases as described by the manufacturer. DNA restriction fragments were separated on 0·7 % (w/v) agarose gels in TAE buffer (0·04 M Tris/acetate, 0·001 M EDTA, pH 8·0). Transfer of DNA to nylon membranes (Hybond-N nylon membrane, Amersham) was carried out as described by the manufacturer. DNA was digoxigenin-labelled and non-isotopic hybridizations were subsequently carried out using the Dig DNA Labeling and Detection Kit (Boehringer Mannheim) as described by the manufacturer.

**DNA transfers.** Transformation of *E. coli* was done by electroporation as described by Dowcet et al. (1988) with the *E. coli* Pulse Apparatus set at 2.5 kV using 0·2 cm gap cuvettes. Conjugations were performed on solidified LB (between *E. coli* strains) or TY (between *E. coli* and *R. meliloti*). After 24 h incubation at 37°C or 30°C respectively, cells were resuspended in saline solution (0·9 g NaCl l–1) and dilutions were plated on selective media.

**Cosmid library construction and screening.** A cosmid DNA library from *R. meliloti* 41 was made as follows. Total DNA was extracted (Meade et al., 1982) and partially digested with BamHI. Phenol/chloroform-purified fragments were dephosphorylated using alkaline phosphatase and subsequently ligated to BamHI-digested cosmid pHC79 or pSF6 at a ratio of 10:1. The ligated DNA was packaged with λ coat proteins by using the Boehringer Mannheim DNA Packaging Kit and transfected into *E. coli* LE392 as described by Hohn (1979) and Hohn & Collins (1980). Cells were plated onto LB-agar containing ampicillin (for pHC79) or spectinomycin (for pSF6). Genomic libraries from *R. meliloti* PHB– (Tn5-induced) mutants (Povolo et al., 1994) were constructed in the same way. After transfection, cells were plated onto LB-agar containing ampicillin and kanamycin to select for inserts bearing the Tn5 insertion. Libraries were screened by hybridization. Colonies were transferred to Hybond-N nylon membranes (Amersham) as described by the manufacturer. DNA was labelled with digoxigenin and hybridization was performed as described above at 68°C with 0·1 x SSC final wash (homologous probes) or at 58°C with 0·5 x SSC final wash (heterologous probes).

**Sequencing and analysis of sequence data.** Dideoxy nucleotide sequencing was carried out from double-stranded DNA using the Applied-Biosystems Taq DyeDeoxy Cycle Sequencing Kit in a thermal cycler with the temperature programme suggested by the manufacturer. An automatic DNA sequencer (model 370A, Applied Biosystmes) was used. Sequences of both strands were obtained from restriction fragments subcloned in pTZ19R using universal primers. Additional sequences were obtained by using custom-made synthetic primers. Sequences were processed and analysed by the IG Molecular Biology Software System, release 5.4 (IntelliGenetics). Homology

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant traits</th>
<th>Reference/origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. meliloti</em> 41*</td>
<td>Wild-type</td>
<td>Prakash et al. (1980)</td>
</tr>
<tr>
<td><em>R. meliloti</em> 4101*</td>
<td>PHB+ mutant of <em>R. meliloti</em> 41</td>
<td>Povolo et al. (1994)</td>
</tr>
<tr>
<td><em>E. coli</em> LE392</td>
<td>Host for cosmid library</td>
<td>Borek et al. (1976)</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>supE supF lacY1 recA1</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1</td>
<td>Mobilizing donor strain, Tra+ pro recA</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSF6</td>
<td>Sp' Sm', Mob', IncW, cos</td>
<td>Selvaraj et al. (1984)</td>
</tr>
<tr>
<td>pTZ19R</td>
<td>Ap', T7-lac tandem promoters, α-lacZ</td>
<td>Mead et al. (1986)</td>
</tr>
<tr>
<td>pTZK19R + KmR8 cassette</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pMP220</td>
<td>Te', lacZ, promoter probe, IncP</td>
<td>Spink et al. (1987)</td>
</tr>
<tr>
<td>pCD1</td>
<td>Cosmid from <em>R. meliloti</em> 41 library in pHC79</td>
<td>This study</td>
</tr>
<tr>
<td>pCD2</td>
<td>Cosmid from <em>R. meliloti</em> 41 library in pHC79</td>
<td>This study</td>
</tr>
<tr>
<td>pTHZ-HX400</td>
<td>phaA, pbaB of <em>R. meliloti</em> 41 in pTZ19R</td>
<td>This study</td>
</tr>
<tr>
<td>pTHZ-ORF1</td>
<td>ORF1 of <em>R. meliloti</em> 41 in pTZ19R</td>
<td>This study</td>
</tr>
<tr>
<td>pS600</td>
<td>phaC of <em>R. meliloti</em> 41 in pMP220</td>
<td>This study</td>
</tr>
<tr>
<td>pTHZ18U-PHB</td>
<td>phaA, pbaB, phaC of <em>A. eutrophus</em> containing insert</td>
<td>E. Dennis, Biology Dept, James Madison University</td>
</tr>
</tbody>
</table>

*The renaming of *Rhizobium meliloti* as *Sinorhizobium meliloti* has recently been proposed (De Lajudie et al., 1994).
searches in the GenBank and SwissProt databases were made with the FASTA and TFASTA programs. Comparisons of amino acid sequences were made with the specific programs included in the PCGENE collection, release 6.0 (IntelliGenetics and Genofit).

**Biochemical analytical procedures.** Cell extracts for enzyme assays were obtained by the following procedure. Samples (300 ml of R. meliloti or 10 ml of E. coli) of late-exponential-phase cultures were centrifuged at 4000g for 15 min at 4 °C. For the β-ketothiolase and acetocetyl-CoA reductase assay, the pellet was resuspended in 2 ml of the following buffer: 100 mM Tris/HCl pH 7.8, 50 mM EDTA, 50 mM β-mercaptoethanol, 0.02 mM PMSF (phenylmethylsulfonyl fluoride), 5% (v/v) glycerol. The suspension was sonicated by using a microprobe-equipped model 450 sonicator (Branson Ultrasonic Corporation) in an ice-water bath for 20 min with pulses of 0.5 s at 50% duty cycle. The extract was centrifuged for 10 min at 10000g at 4 °C. The supernatant was kept on ice until analysis. Protein content was quantified by using the Coomassie Plus Protein Assay Reagent Kit (Pierce) calibrated with bovine serum albumin. The β-ketothiolase (thiolysis reaction) and acetocetyl-CoA reductase assays were conducted as described by Senior & Dawes (1971) and Haywood et al. (1988), respectively. PHB content in bacterial cells was measured as described by Braunegg et al. (1978). A model 8420 capillary gas-chromatograph (Perkin-Elmer) with a WCOT fused silica capillary column and CP-WAX-58 CB liquid phase (Chrompack) was used. Working conditions have already been reported (Povolo et al., 1994).

**RESULTS AND DISCUSSION**

**Cloning and sequencing of phaA and phaB**

A cosmid genomic library of R. meliloti 41 in pHC79 was screened by colony hybridization with a probe containing the phaC, phaA and phaB genes from Alcaligenes eutrophus (Slater et al., 1988). Under low-stringency conditions two clones were isolated, containing plasmids pCDP1 and pCDP2. Plasmid pCDP1 contained three BamHI fragments of about 4300, 1700 and 300 bp respectively which hybridized with the A. eutrophus probe. pCDP2 shares with pCDP1 the 4300 bp BamHI hybridizing fragment. In Fig. 1(a) the physical and genetic map of the cloned region, as determined by nucleotide sequencing, is shown. The 4300 bp BamHI fragment common to pCDP1 and pCDP2 is the one between the first two BamHI sites on the left in Fig. 1(a), including ORF1 and part of phaA.

**Analysis of the sequence containing phaA and phaB**

The presence of the phaA and phaB genes in the sequenced region was indicated on the basis of DNA homology. However pCDP1, containing the sequenced region, conferred neither β-ketothiolase nor acetocetyl-CoA reductase activities upon E. coli (see Table 4). Amino acid sequences deduced from the ORFs of the region showing homology with phaA and phaB genes were compared with those from published nucleotide sequences of β-ketothiolase and acetocetyl-CoA reductase (Tables 2 and 3). In Table 2, as well as sequences of β-ketothiolases from PHB producers (shown in bold type), some from non-PHB-producing organisms have been included, to point out the much higher level of homology found between the sequenced phaA gene and members of the former group. It is apparent that the sequenced region of R. meliloti codes for these two enzymes. The products of phaA and phaB exhibit homology ranging from 59 to 68% with corresponding gene products in A. eutrophus, C. vinous and T. violacea, and a remarkably high homology with the corresponding proteins in Z. ramigera (88 and 86.7% identity for β-ketothiolase and acetocetyl-CoA reductase respectively). However, the high DNA homology with the corresponding genes in Z. ramigera extends into the phaA upstream region (Fig. 2) covering the putative promoter structure. In R. meliloti the distance from this site and the phaA translational start point is about 50 nucleotides shorter. The bacterial strain known as 1-16-M was originally identified as Zoogloea ramigera but it is clear from analysis of 16S ribosomal RNA sequences (Shin et al., 1993) that the strain does not belong to the genus Zoogloea. Strain 1-16-M is in fact related to Agrobacterium and Rhizobium, although its exact identity has not been established. Our results indeed suggest a close relation between Rhizobium and 'Z. ramigera' 1-16-M.

Another open reading frame (ORF1) immediately upstream of the phaA–phaB genes, but coded in the opposite direction, is homologous to a sequence (ORF4) related to PHB biosynthesis in Chromatium vinous and Thioctysist violacea (Liebergessell & Steinbüchel, 1992, 1993). In these photosynthetic bacteria ORF4 is located between the phaA and phaB genes. Comparison of the two amino acid sequences by the method proposed by Needleman & Wunsch (1970) gave an alignment score of 138. This value is considerably higher than the score (i.e. 3) often used as cut-off point to indicate probable relatedness. In Fig. 3 the amino acid sequences of ORF1 in R. meliloti and ORF4 in C. vinous are compared. The results show an identity of 40.5% and a similarity of 11.8%. Beyond the stop codon of ORF1 in R. meliloti there are 10 amino acids showing remarkable homology with the last amino acids of ORF4 of C. vinous. It could be inferred that the stretch of residues from positions 151 and 192 in ORF1, having no match in the corresponding C. vinous sequence, arose from a DNA insertion bearing a stop codon at position 194. A codon usage analysis was carried out for ORF1 to compare frequencies with those of 18 R. meliloti genes in the GenBank database. The data matched the most frequent choices on almost all residues, suggesting a genuine coding nature for the frame identified. Fur-
The table shows the percentage of identical and similar (in parentheses) amino acids of the known \( \beta \)-ketothiolases (EC 2.3.1.16) (f, i) or acetoacetyl-CoA thiolases (EC 2.3.1.9) (a, b, c, d, e, g, h, i, k, l). ‘Similar’ indicates those residues which can be generally interchanged without modifying the conformation of a protein, that is: A-S-T; D-E; N-Q; R-K; I-L-M-V; F-Y-W. The amino acid sequences have been deduced from published nucleotide sequences of \( \textit{Zoogloea ramigera} \) (Peoples et al., 1987), \( \textit{Alcaligenes eutrophus} \) (Peoples & Sinskey, 1989b), \( \textit{Chromatiurn vinosum} \) (Liebergessell & Steinbuechel, 1992), \( \textit{Thiorysitis violacea} \) (Liebergessell & Steinbuechel, 1993), \( \textit{Escherichia coli} \) (Yang et al., 1990), \( \textit{Rattus norvegicus} \) (Arakawa et al., 1987), \( \textit{Sachrothomes varius} \) (Dequinn et al., 1988), \( \textit{Candida tropicalis} \) (Kurihara et al., 1992), \( \textit{Sachrothomes terrisiae} \) (Hiser et al., 1993), \( \textit{Raphanus sativus} \) (Vollack et al., 1994) and \( \textit{Homo sapiens} \) (Fukao et al., 1990). The data in bold type are from PHB-producing organisms.

### Table 2. Homology of \( \beta \)-ketothiolases

<table>
<thead>
<tr>
<th></th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
<th>(e)</th>
<th>(f)</th>
<th>(g)</th>
<th>(h)</th>
<th>(i)</th>
<th>(j)</th>
<th>(k)</th>
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</thead>
<tbody>
<tr>
<td>R. meliloti</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Z. ramigera</td>
<td>86.7</td>
<td>65.1</td>
<td>58.3</td>
<td>50.0</td>
<td>42.3</td>
<td>35.0</td>
<td>28.9</td>
<td>23.0</td>
<td>18.0</td>
<td>14.0</td>
<td>10.0</td>
</tr>
<tr>
<td>A. eutrophus</td>
<td>65.1</td>
<td>50.0</td>
<td>42.3</td>
<td>35.0</td>
<td>28.9</td>
<td>23.0</td>
<td>18.0</td>
<td>14.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
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<tr>
<td>C. vinsonii</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Data are expressed as in Table 2. The sources of nucleotide sequences are the same as in Table 2 except for \( \textit{Zoogloea ramigera} \) (Peoples & Sinskey, 1989).

### Table 3. Homology of acetoacetyl-CoA reductase

Data are expressed as in Table 2. The two ORFs display 37.3% identity and 118% similarity. The 10 hypothetical residues past the stop codon (end) in \( \textit{R. meliloti} \) ORF1 are reported to show homology with the C-terminal part of the \( \textit{C. vinsonii} \) ORF4 product.

### Fig. 2. Alignment of the nucleotide sequences of the corresponding \(-10\) and \(-35\) putative promoter (in boldface) regions of the \(\textit{phaA}-\textit{phaB}\) operon in \(\textit{Zoogloea ramigera}\) (Peoples & Sinskey, 1989) with the homologous \(\textit{R. meliloti}\) sequence. The asterisk indicates the transcriptional start site in \(\textit{Z. ramigera}\).

### Fig. 3. Alignment of the amino acid sequences of ORF4 of \(\textit{Chromatiurn vinosum}\) and ORF1 of \(\textit{R. meliloti}\) deduced from the nucleotide sequences. The two ORFs display 37.3% identity and 118% similarity. The 10 hypothetical residues past the stop codon (end) in \(\textit{R. meliloti}\) ORF1 are reported to show homology with the C-terminal part of the \(\textit{C. vinsonii}\) ORF4 product.

### Fig. 4. Alignment of the nucleotide sequences of the corresponding \(-10\) and \(-35\) putative promoter (in boldface) regions of the \(\textit{phaA}-\textit{phaB}\) operon in \(\textit{Zoogloea ramigera}\) (Peoples & Sinskey, 1989) with the homologous \(\textit{R. meliloti}\) sequence. The asterisk indicates the transcriptional start site in \(\textit{Z. ramigera}\).

thermore ORF1 was shown to be exessible in \(\textit{E. coli}\) when cloned in pTZ19R, giving rise to an additional protein band of about 22 kDa, when cell lysates of
**Table 4. Expression of phaA and phaB**

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Ketothiolase*</th>
<th>Acetoacetyl-CoA reductase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109(pTZ-HX400)</td>
<td>0.13</td>
<td>0.29</td>
</tr>
<tr>
<td>E. coli JM109(pTZ19R)</td>
<td>0.007</td>
<td>0.00</td>
</tr>
<tr>
<td>R. meliloti 41</td>
<td>1.2</td>
<td>0.047</td>
</tr>
<tr>
<td>E. coli LE392(pCDP1)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Specific activity: units (mg protein)⁻¹ [1 unit equals the amount of enzyme consuming 1 μmol acetoacetyl-CoA min⁻¹ (β-ketothiolase) or 1 μmol NADPH min⁻¹ (acetoacetyl-CoA reductase)].

![Fig. 4. Physical and genetic map of the cloned region containing phaC. The solid line represents the sequenced DNA. Dotted lines encompass regions not drawn to scale. S, Sm:; Smal; B, BamHI; Sp, Sphi; Sa, Sall; H, HindIII; X, Xhol; A, Avil; E, EcoRI. The Smal–SacI fragment labelled S600 was used for complementation studies. The TnS insertion site in mutant 41011 is indicated.](image)

Km-resistant derivative of pTZ19R, and introduced into E. coli JM109 containing phaC cloned in pTZ19R. Gas chromatographic analyses proved that the co-presence of β-ketothiolase, reductase and synthase led to the production of small but measurable amounts of PHA in E. coli when grown in the presence of 20 g glucose l⁻¹ (data not shown). Controls containing either the phaC gene alone or the pTZ19R vector did not reveal polymer accumulation. These results confirm that phaA and phaB are necessary for PHA production.

**Cloning and sequencing of phaC**

A genomic cosmid library from R. meliloti 41011, a PHB⁺ mutant of R. meliloti 41 obtained by Tn5 mutagenesis (Povolo et al., 1994), was made in pHC79. Using an internal HindIII fragment from Tn5 as a probe, a positive clone was detected by colony hybridization. Since Tn5 has no EcoRI sites and one BamHI site, regions flanking the insertion were identified in an EcoRI–BamHI digest of the cosmid by hybridization with Tn5 and used as a probe to screen a pSF6 cosmid genomic library from the parental R. meliloti 41. A positive clone was identified and the restriction map of the region of interest was determined (Fig. 4); 2653 bases were sequenced.

**Nucleotide sequence analysis of phaC**

The sequence analysis showed the presence of the phaC gene on the basis of DNA homology with published sequences (Hustede et al., 1992; Valentin & Steinbüchel, 1993; Pieper & Steinbüchel, 1992; Liebergesell & Steinbüchel, 1993). The highest homology at the amino acid level was found with the methylotrophic bacterium *Mutithromobacter extorquens* (Valentin & Steinbüchel, 1993). The PHB synthase of *R. meliloti* 41 matches the consensus for those amino acid residues present in all PHB synthases involved in the biosynthesis of short-chain hydroxyalkanoic acids (Steinbüchel et al., 1992). As the N-termini of the synthases show poor homology, the start codon was assigned on the basis of length comparison with other PHB synthases. An alternative but less likely GUG start codon lies 102 bp upstream of the proposed one.

**Complementation studies of phaC**

A 3 kb Smal–SacI fragment containing the phaC gene was blunt-ended at the SacI terminus by Klenow DNA polymerase and subcloned into a blunt-ended BamHI site of the broad-host-range vector pMP220 yielding pS600 which, when transferred to *R. meliloti* 41011, could complement the mutant’s complete inability to accumulate PHB. The introduced plasmid led to the production of 31.9% (w/w of bacterial dry weight) PHB, a value close to the wild-type yield of 36.2%. Since pMP220 is a promoter-probe vector designed for LacZ assays and therefore devoid of any promoters acting on the polycloning site region, the expression of phaC which, moreover, occurs in both cloning orientations, indicates the presence of its own promoter within the 6 kb SacI fragment. This conclusion is further supported by the fact that β-galactosidase activity is obtained in *R. meliloti* only when the phbC-containing fragment is cloned in the same orientation with respect to the reporter lacZ gene. The same construct, however, does not confer a LacZ⁺ phenotype upon *E. coli* JM109, suggesting that the *R. meliloti* phaC promoter is not functional in this background.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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