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

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Genes encoding β -ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*) and PHB-synthase (*phaC*) from *R. meliloti* 41, together with a fourth gene, referred to as ORF1, presumed to be involved in PHB biosynthesis, have been cloned and sequenced. *phaA*, *phaB* and ORF1 were identified by heterologous hybridization on a cosmid library, while *phaC* was isolated by cloning the transposon-tagged fragment from a *R. meliloti* PHB⁻ Tn5 mutant. *phaA* and *phaB* were functionally expressed in *Escherichia coli* while *phaC* was able to complement a PHB⁻ strain of *R. meliloti* 41. The three genes were sufficient to direct the production of polyhydroxyalkanoate in *E. coli*. The homology of ORF1 with an ORF located near the PHB genes in two phototrophic bacteria suggests its involvement in PHB synthesis.

Keywords: *Rhizobium meliloti*, poly- β -hydroxybutyrate, *phaA*, *phaB*, *phaC*

INTRODUCTION

In the last two decades much research has been done on polyesters synthesized and accumulated by micro-organisms 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* (PHB 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* (Liebergesell & Steinbüchel, 1992) and *Acinetobacter* sp. (Schembri *et al.*, 1994a, b); *phaA*, *C* from *Thiocystis violacea* (Liebergesell & Steinbüchel, 1993); *phaC* from *Rhodobacter sphaeroides* (Hustede *et al.*, 1992; Hustede & Steinbüchel, 1993), *Methylobacterium extorquens* (Valentin & Steinbüchel, 1993) and *Rhodococcus ruber* (Pieper & Steinbüchel, 1992); and *phaA*, *B* from *Zoogloea 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 (Liebergesell & Steinbüchel, 1992, 1993; Liebergesell *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 (Pries *et al.*, 1991, 1992) and the

Abbreviations: PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate.
The GenBank accession numbers for the sequences reported in this paper are U17226 and U17227.

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, 0.9 g CaCl₂·2H₂O;

pH 6.8) or RMM medium (per litre: 2.05 g K₂HPO₄, 1.45 g KH₂PO₄, 0.50 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 biotin, 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 (Qiaex, 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 Dower *et al.* (1988) with the *E. coli* Pulser 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 *Bam*HI. Phenol/chloroform-purified fragments were dephosphorylated using alkaline phosphatase and subsequently ligated to *Bam*HI-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 × SSC final wash (homologous probes) or at 58 °C with 0.5 × 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 Biosystems) 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

Strain or plasmid	Relevant traits	Reference/origin
Bacteria		
<i>R. meliloti</i> 41*	Wild-type	Prakash <i>et al.</i> (1980)
<i>R. meliloti</i> 41011*	PHB ⁻ mutant of <i>R. meliloti</i> 41	Povolo <i>et al.</i> (1994)
<i>E. coli</i> LE392	Host for cosmid library	Borck <i>et al.</i> (1976)
<i>E. coli</i> JM109	<i>supE supF lacY1 recA1</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> S17-1	Mobilizing donor strain, Tra ⁺ <i>pro recA</i>	Simon <i>et al.</i> (1983)
Plasmids		
pHC79	Ap ^r , cos	Hohn & Collins (1980)
pSF6	Sp ^r Sm ^r , Mob ⁺ , IncW, cos	Selvaraj <i>et al.</i> (1984)
pTZ19R	Ap ^r , T7- <i>lac</i> tandem promoters, α- <i>lacZ</i>	Mead <i>et al.</i> (1986)
pTZK19R	pTZ19R + Km ^R cassette	This study
pMP220	Tc ^r , 'lacZ, promoter probe, IncP	Spaink <i>et al.</i> (1987)
pCDP1	Cosmid from <i>R. meliloti</i> 41 library in pHC79	This study
pCDP2	Cosmid from <i>R. meliloti</i> 41 library in pHC79	This study
pTZ-HX400	<i>phaA</i> , <i>phaB</i> of <i>R. meliloti</i> 41 in pTZ19R	This study
pTZ-ORF1	ORF1 of <i>R. meliloti</i> 41 in pTZ19R	This study
pS600	<i>phaC</i> of <i>R. meliloti</i> 41 in pMP220	This study
pTZ18U-PHB	<i>phaA</i> , <i>phaB</i> , <i>phaC</i> of <i>A. eutrophus</i> containing insert	E. Dennis, Biology Dept, James Madison University

* 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 4000 *g* for 15 min at 4 °C. For the β -ketothiolase and acetoacetyl-CoA reductase assay, the pellet was resuspended in 2 ml of the following buffer: 10.0 mM Tris/HCl pH 7.88, 5.0 mM EDTA, 5.0 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 10000 *g* 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 acetoacetyl-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 Braune *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 *Bam*HI fragments of about 4300, 1700 and 300 bp respectively which hybridized with the *A. eutrophus* probe. pCDP2 shares with pCDP1 the 4300 bp *Bam*HI 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 *Bam*HI fragment common to pCDP1 and pCDP2 is the one between the first two *Bam*HI sites on the left in Fig. 1(a), including ORF1 and part of *phaA*.

Fig. 1. (a) Physical and genetic map of the cloned region containing the *phaA* and *phaB* genes and ORF1. The solid line represents the sequenced DNA. Dotted lines encompass region not drawn to scale. B, *Bam*HI; S, *Sac*I; P, *Pst*I; H, *Hind*III; E, *Eco*RI; X, *Xho*I. (b) Subcloning strategy. Fragments labelled pTZORF1 and HX400 were used in expression studies.

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 acetoacetyl-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 acetoacetyl-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. vinosum* and *T. violacea*, and a remarkably high homology with the corresponding proteins in *Z. ramigera* (88 and 86.7% identity for β -ketothiolase and acetoacetyl-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 I-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 I-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' I-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 vinosum* and *Thiocystis violacea* (Liebergesell & 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 13.8. 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. vinosum* 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. vinosum*. It could be inferred that the stretch of residues from positions 151 and 192 in ORF1, having no match in the corresponding *C. vinosum* 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-

Table 2. Homology of β -ketothiolases

The data show the percentage of identical and similar (in parentheses) amino acids of the known β -ketothiolases (EC 2.3.1.16) (f, j) 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 *Zoogloea ramigera* (Peoples *et al.*, 1987), *Alcaligenes eutrophus* (Peoples & Sinskey, 1989b), *Chromatium vinosum* (Liebergesell & Steinbüchel, 1992), *Thyocystis violacea* (Liebergesell & Steinbüchel, 1993), *Escherichia coli* (Yang *et al.*, 1990), *Rattus norvegicus* (Arakawa *et al.*, 1987), *Saccharomyces uvarum* (Dequinn *et al.*, 1988), *Candida tropicalis* (Kurihara *et al.*, 1992), *Saccharomyces cerevisiae* (Hiser *et al.*, 1993), *Raphanus sativus* (Vollack *et al.*, 1994) and *Homo sapiens* (Fukao, *et al.*, 1990). The data in bold type are from PHB-producing organisms.

		(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)
<i>R. meliloti</i>	(a)	100	88.0 (5.1)	63.4 (11.7)	62.6 (14.0)	61.3 (14.8)	43.4 (13.4)	43.3 (14.0)	42.7 (13.7)	40.2 (15.5)	41.7 (17.6)	42.5 (15.5)	39.4 (14.8)
<i>Z. ramigera</i>	(b)		100	63.2 (11.5)	60.4 (16.1)	59.6 (16.1)	41.9 (12.4)	41.2 (15.6)	40.4 (15.6)	40.2 (14.1)	39.6 (17.6)	41.4 (15.9)	34.3 (16.1)
<i>A. eutrophus</i>	(c)			100	67.9 (13.2)	68.7 (13.5)	42.9 (15.8)	43.8 (15.0)	45.0 (14.0)	45.3 (13.7)	41.5 (17.6)	44.8 (15.5)	39.4 (13.2)
<i>C. vinosum</i>	(d)				100	87.3 (6.6)	44.4 (12.4)	43.1 (16.8)	43.7 (15.7)	42.6 (15.7)	41.6 (17.5)	39.6 (18.3)	38.8 (14.2)
<i>T. violacea</i>	(e)					100	45.0 (13.7)	40.9 (17.0)	41.4 (16.0)	42.6 (15.5)	41.6 (17.0)	40.9 (18.3)	37.1 (15.7)
<i>E. coli</i>	(f)						100	33.1 (13.2)	33.3 (12.9)	37.0 (14.0)	39.3 (10.3)	34.6 (14.7)	31.8 (9.8)
<i>S. uvarum</i>	(g)							100	95.5 (2.8)	65.6 (13.3)	38.8 (14.9)	49.5 (13.6)	46.5 (13.1)
<i>S. cerevisiae</i>	(h)								100	66.6 (12.8)	39.5 (14.1)	49.2 (13.6)	47.0 (13.3)
<i>C. tropicalis</i> (peroxisomal)	(i)									100	34.8 (18.1)	48.4 (14.1)	46.9 (14.4)
<i>R. norvegicus</i> (mitochondrial)	(j)										100	38.8 (15.6)	33.3 (12.3)
<i>H. sapiens</i> (liver, mitochondrial)	(k)											100	43.8 (14.6)
<i>R. sativus</i> (seedling)	(l)												100

Table 3. Homology of acetoacetyl-CoA reductase

Data are expressed as in Table 2. The sources of nucleotide sequences are the same as in Table 2 except for *Z. ramigera* (Peoples & Sinskey, 1989).

		(a)	(b)	(c)	(d)
<i>R. meliloti</i>	(a)	100.0	86.7 (6.6)	51.0 (13.3)	50.6 (14.1)
<i>Z. ramigera</i>	(b)		100.0	52.3 (13.3)	51.5 (14.1)
<i>A. eutrophus</i>	(c)			100.0	56.1 (14.6)
<i>C. vinosum</i>	(d)				100.0

R.m.	GCAACTAGCCGTTTGA	CTCGCGCTCCTCC-CTTTGCCAGTCT	-----GC	43
Z.r.	GGGATATCCCGTTTGA	CTCTCCGCTTTGCCAGTTT	GATTTCGGGAGAACCGC	59
R.m.	C-----	TCCAT--CAGGCA--AAG-----CAAAT		63
Z.r.	CGGGCTCGCGCGGTT	CGTGC	CGGACCGGTTTCCAGGCGCGCGCAAGGCTGCCAGAA	119
R.m.	ATGAGGAACGTCCCAT	GAGCAATCCCTCGATCGTCATCGCCAGCGCGGCTCGCACGGCGG		123
Z.r.	CTGAGGAAGCAGATAT	GAGCACCCGTCATCGTCATCGCCAGCGC---CGGACCGCGG		176

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

thermore ORF1 was shown to be expressible in *E. coli* when cloned in pTZ19R, giving rise to an additional protein band of about 22 kDa, when cell lysates of

RMORF1	MGKNEGQIVIKKYANRRRLVNTGTSTYVTLDDLAVMKRGEDFIQDAKSGE	51
CVIORF4	MNSERI---IKKYPNRLYDTEVSRVITLADVRDLVMSGQPFVRVLDSSANDS	48
RMORF1	DITHSVLTQIIIFEQESKNTGNTLLPISFLRLQISFYGDQMQMVVPSYLEHSM	102
CVIORF4	DITRSILLQIMLEEETG-GQPLFSANMLAQIRFYGGTLQGTFFARYLESSL	98
RMORF1	QAFTEQQAQMREQITKAFGDTPIGKLNKVLPLVLEEQVRRNTEMFHQAMQM	153
CVIORF4	DLFAKQQ---QEVTKALTDNPFGTVTRLTQKNVEIWADLQDELMRAA---	142
RMORF1	FSFFMAAPPKAKETRKAEAKDIDELKEQRLALQTKLDNLGendSPLDPPKKGK	204
CVIORF4	-----G---FPVAPRKKE	153

Fig. 3. Alignment of the amino acid sequences of ORF4 of *Chromatium vinosum* and ORF1 of *R. meliloti* deduced from the nucleotide sequences. The two ORFs display 37.3% identity (:) and 11.8% similarity (.). The 10 hypothetical residues past the stop codon (end) in *R. meliloti* ORF1 are reported to show homology with the C-terminal part of the *C. vinosum* ORF4 product.

JM109(pTZ19R) and JM109(pTZORF1) were compared by SDS-PAGE. The molecular mass observed was in agreement with that expected from the deduced amino acid sequence of ORF1.

Heterologous expression of *phaA* and *phaB*

β -Ketothiolase and acetoacetyl-CoA reductase activities could not be detected in *E. coli* containing pCDP1, nor was PHB found (Table 4). In contrast, when the fragment indicated as HX400 in Fig. 1(b) was subcloned in pTZ19R with the *phaA/B* genes downstream of the T7-*lac* tandem vector promoters, both activities could be detected in *E. coli* JM109. In order to prove the involvement of *phaA* and *phaB* in PHA synthesis, the two cloned genes carried by fragment HX400 were subcloned in pTZK19R, a

Table 4. Expression of *phaA* and *phaB*

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

* Specific activity: units (mg protein)⁻¹ [1 unit equals the amount of enzyme consuming 1 μ mol acetoacetyl-CoA min⁻¹ (β -ketothiolase) or 1 μ mol 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, *SacI*; Sm, *SmaI*; B, *BamHI*; Sp, *SphI*; Sa, *SalI*; H, *HindIII*; X, *XhoI*; A, *AvaI*; E, *EcoRI*. The *SmaI*–*SacI* fragment labelled 5600 was used for complementation studies. The Tn5 insertion site in mutant 41011 is indicated.

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 *Methylobacterium 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 *SmaI*–*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 poly-cloning 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.

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