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Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*

Matthias Liebergesell, Alexander Steinbüchel

Institut für Mikrobiologie, Georg-August-Universität Göttingen, Grisebachstrasse 8, W-3400 Göttingen, Federal Republic of Germany

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Abstract. From a genomic library of *Thiocystis violaceae* strain 2311 in λL47, two adjacent *Eco*RI restriction fragments of 5361 base pairs (bp) and of 1978 bp were cloned. The 5361-bp *Eco*RI restriction fragment hybridized with a DNA fragment harbouring the *Alcaligenes eutrophus* poly(3-hydroxyalkanoate) (PHA) synthase operon (*phbCAB*) and restored the ability to synthesize and accumulate PHA in PHA-negative mutants derived from *A. eutrophus*. The nucleotide sequence analysis of both fragments revealed five open-reading frames (ORFs); at least three of them are probably relevant for PHA biosynthesis. The amino acid sequences of the putative proteins deduced from these genes indicate that they encode a β-ketothiolase [$phbA_{Tv}$, relative molecular mass (M_r) 40850], which exhibited 87.3% amino acid identity with the β-ketothiolase from *Chromatium vinosum*. The amino acid sequences of the putative proteins deduced from $ORF2_{Tv}$ (M_r 41450) and $phbC_{Tv}$ (M_r 39550), which were located upstream of and antilinear to $phbA_{Tv}$, exhibited 74.7% and 87.6% amino acid identity, respectively, with the corresponding gene products of *C. vinosum*. Downstream of and antilinear to $phbC_{Tv}$ was located ORF5, which encodes for a protein of high relative molecular mass (M_r 76428) of unknown function. With respect to the divergent organisation of $ORF2_{Tv}$ and $phbC_{Tv}$ on one side and of $phbA_{Tv}$ on the other side and from the homologies of the putative gene products, this region of the *T. violaceae* genome resembled very much the corresponding region of *C. vinosum*, which was identified recently.

Introduction

Polyhydroxyalkanoic acids (PHA) are accumulated by many bacteria if a carbon source is provided in excess and if one nutrient, which is essential for growth, has been deplete (reviewed by Anderson and Dawes 1990). A wide variety of almost 40 different hydroxyalkanoic acids has been already identified as constituents of these polyesters (Steinbüchel 1991). As these polyesters are thermoplastic and biodegradable and as they can be obtained from renewable resources, PHA are of biotechnological interest; some polyesters are already produced on an industrial scale (Byrom 1990), and the metabolism of PHA is currently investigated in many laboratories.

Four principle PHA-biosynthetic pathways, each with modifications, may account for the synthesis of these polyesters in different bacteria (Steinbüchel and Schlegel 1991; Steinbüchel 1991). Much progress has been made in the last few years in the molecular analysis of genes that are relevant for the biosynthesis of PHA. Whereas in *Alcaligenes eutrophus* the structural genes for biosynthetic β-ketothiolase ($phbA_{Ae}$), NADPH-dependent acetoacetyl-CoA reductase ($phbB_{Ae}$) and PHB synthase ($phbC_{Ae}$) are organized in one signal operon as $phbCAB_{Ae}$ (reviewed by Steinbüchel and Schlegel 1991), only $phbA_{Zr}$ and $phbB_{Zr}$ are clustered in *Zoogloea ramigera*; $phbC_{Zr}$ has not been identified yet in the latter bacterium (Peoples and Sinskey 1989). *Pseudomonas oleovorans* possesses probably two different PHA synthase genes, which are represent by open-reading frame ORF1 and ORF3 and which flank a gene for a PHA depolymerase represented by ORF2 (Huisman et al. 1991). Anoxygenic photosynthetic bacteria are well known for their ability to accumulate large amounts of PHA (for a recent survey see Liebergesell et al. 1991). Recently, we cloned the PHA-biosynthetic genes of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*. The genes of the latter bacterium conferred the ability to form extraordinarily large PHB granules to a PHA-negative mutant of *A. eutrophus* (Hustede et al. 1992). In *Chromatium vinosum* we identified at least four genes relevant for PHA biosynthesis on one single genomic DNA fragment, which also conferred the ability to synthesize PHA to other bacteria. Detailed analysis revealed that these genes are transcribed divergently from at least two different overlapping promoters (Liebergesell and Steinbüchel 1992). These studies provided also first evidence that additional ORFs are required for PHA biosynthesis

Correspondence to: A. Steinbüchel

in *C. vinosum*. In this study, we cloned and analysed PHA-biosynthetic genes from *Thiocystis violaceae* and confirmed the unusual organisation of the corresponding genes in *C. vinosum*.

Materials and methods

Bacterial strains, plasmids and bacteriophage. The bacterial strains used in this study were *T. violaceae* 2311 (DSM 208), *A. eutrophus* H16 (DSM 428; ATCC 17699), *A. eutrophus* PHB$^-$4 (PHB-negative mutant of H16; DSM 541), *A. eutrophus* H1479 (PHB-leaky mutant of a Sm^r-strain of H16; Pries et al. 1991), *Escherichia coli* S17-1 (Simon et al. 1983), *E. coli* WL87 (Amersham Buchler, Braunschweig, FRG), *E. coli* XL1-Blue (Bullock et al. 1987). The bacteriophage λL47 was described by Loenen and Brammar (1980). The plasmids used in this study were pHP1016 (Pries et al. 1991), pBluescript SK$^-$ (Stratagene, San Diego, Calif., USA), pSK2665 (pBluescript KS$^-$::SE52, *phbCAB*$_{Ae}$; Schubert et al. 1991), pSE45 (pBluescript KS$^-$::SE45, *phbA*$_{Cv}$, *phbC*$_{Cv}$, ORF2 of *C. vinosum*; Liebergesell and Steinbüchel 1992), pHP1016::E54 (this study), pE54 (this study), pE20 (this study).

Growth of bacteria. E. coli was grown at 37° C in Luria-Bertani (LB) medium, whereas *A. eutrophus* was grown aerobically at 30° C in either a nutrient broth (NB) medium (0.8%, w/v) or in a mineral salts medium (MM), which was supplemented with filter-sterilized carbon sources as indicated in the text (Schlegel et al. 1961). To allow extensive accumulation of PHB, the concentration of NH_4Cl in the MM was reduced to 0.05 or 0.005% (w/v), and 0.5% (w/v) glucose was added to the LB medium. *T. violaceae* was cultivated under anaerobic conditions in the light in 100-ml screw-capped bottles at a light intensity of approximately 1000 lx in the medium described by Biebl and Pfennig (1978), which contained 20 mM acetate.

Analysis of PHA. To determine the PHA content of the cells and the composition of the polyester, PHA of 4±1 mg freeze-dried cells was transformed to the constituent 3-hydroxycarboxylic acid methyl esters by methanolysis (Brandl et al. 1988). These esters were analysed by gas chromatography as described recently in detail (Brandl et al. 1988; Timm et al. 1990).

Optical rotation of methyl 3-hydroxybutyric acid was measured in chloroform solution at 589 nm in a Perkin Elmer Polarimeter 241 apparatus according to Haywood et al. (1991). Methyl esters were prepared by incubation of 40 mg polyester for 3.5 h at 100° C in sealed tubes with 2 ml solution of 3% (v/v) sulphuric acid in redistilled chloroform. After cooling, the organic phase was extracted with 1 ml water, the methyl esters were recovered from the chloroform phase by evaporation, and the esters were dissolved in redistilled chloroform at a concentration of 15 mg/ml for measurement.

Determination of enzyme activities. The activities of β-ketothiolase (EC 2.3.1.9) and of NADPH- or NADH-dependent acetoacetyl-CoA reductase (EC 1.1.1.36 and EC 1.1.1.35, respectively) were determined in the soluble fractions by optical tests. Activity of PHA synthase was determined in crude cellular extracts by a radiometric assay (Steinbüchel et al. 1992). Soluble fractions and crude cellular extracts were obtained and assays were performed as described by Schubert et al. (1988). Soluble protein was determined as described by Lowry et al. (1951).

Isolation and analysis of deoxynucleic acid. Isolation and manipulation of DNA, construction of a *T. violaceae* genomic library in λL47, hybridization experiments employing biotinylated DNA probes, synthesis of oligonucleotides from deoxynucleoside phosphoamidites, and DNA sequencing by the dideoxy chain termination method with alkaline-denaturated double-stranded plasmid DNA were done as described previously (Liebergesell and Steinbüchel 1992).

Transfer of DNA. Transformation of *E. coli* was done by the $CaCl_2$ procedure as described by Hanahan (1983). Matings of *A. eutrophus* (recipient) with *E. coli* S17-1 (donor) harbouring hybrid plasmids were performed on solidified NB medium as described by Friedrich et al. (1981).

Analysis of sequence data. Nucleic acid sequence data and deduced amino acid sequences were analysed with the Genetic Computer Group Sequence Analysis Software Package (GCG Package, version 6.2, June 1990) as described by Devereux et al. (1984).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported here have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession no. 01113.

Results

Identification and cloning of a genomic fragment relevant for biosynthesis of PHA

A 5.2-kilo base pair (kbp) *Sma*I/*Eco*RI-restriction fragment (SE52), which harboured the *A. eutrophus* structural genes for β-ketothiolase (*phbA*$_{Ae}$), NADPH-dependent acetoacetyl-CoA reductase (*phbB*$_{Ae}$) and PHA synthase (*phbC*$_{Ae}$) (for review see Steinbüchel and Schlegel 1991), was used as a heterologous hybridization probe to detect homologous genes of *T. violaceae*. If *Eco*RI-digested genomic DNA of *T. violaceae* was separated by agarose gel electrophoresis and hybridized with biotinylated SE52 DNA, one single signal representing a 5.4-kbp restriction fragment appeared. A strong signal of identical size appeared if the 4.5-kbp *Sma*I/*Eco*RI fragment SE45 of *C. vinosum*, which harboured genes for PHA synthase (*phbC*$_{Cv}$), β-ketothiolase (*phbA*$_{Cv}$) and ORF2 (Liebergesell and Steinbüchel 1992), was used as a probe.

Biotinylated SE52 DNA was used to detect the 5.4-kbp *Eco*RI-restriction fragment in a λL47 gene bank of *T. violaceae* in *E. coli* WL87, which derived from incompletely *Eco*RI-digested genomic DNA. Ten plaques among 600 tested gave a hybridization signal, and one positive recombinant phage was purified to homogeneity. This phage harboured three genomic DNA fragments of 5.4, 5.2 and 2.0 kbp. Biotinylated SE52 DNA and a subfragment thereof, which harboured only *phbA*$_{Ae}$ and *phbB*$_{Ae}$, hybridized with the 5.4-kbp *Eco*RI fragment; in contrast, a subfragment, which harboured solely *phbC*$_{Ae}$, gave no signal. Hybridization experiments employing the 5.4- and the 2.0-kbp fragment, which were referred to as E54 or E20, respectively, revealed that both fragments are linked in the genome of *T. violaceae*.

Complementation and heterologous expression

Fragment E54 was cloned in the *Eco*RI-site of the broad-host-range vector pHP1016. Plasmid

Table 1. Analysis of activities of *Thiocystis violaceae* polyhydroxyalkanoic acid (PHA)-biosynthetic enzymes, and heterologous expression in *Escherichia coli* and in *Alcaligenes eutrophus* H16-PHB$^-$4[a]

Strain (plasmid)	Relevant markers	Medium	Specific activity (U/g of protein)[b]				
			PHA synthase	β-Keto-thiolase	Acetoacetyl-CoA reductase		Accumulation of PHB
					NADH-dependent	NADPH-dependent	(% of cellular dry matter)
T. violaceae	*phbA*$^+$, *phbB*$^+$, *phbC*$^+$, ORF2$^+$	MM-Ac	10.6	1395	516	110	83.0
A. eutrophus							
H16	*phbA*$^+$, *phbB*$^+$, *phbC*$^+$	MM-Fruc	10.1	1980	940	740	80.5
PHB$^-$4	*phbA*$^-$, *phbB*$^-$, *phbC*$^-$	MM-Fruc	<0.1	660	1250	130	<0.1
PHB$^-$4 (pHP1016::E54)	*phbA*$^+$, *phbB*$^-$, *phbC*$^+$, ORF2$^+$	MM-Tc-Fruc	3.7	622	1120	94	66.1
E. coli							
S17-1 (pHP1016)	None	LB-Tc-Glu	<0.1	<20	<20	<20	<0.1
S17-1 (pHP1016::E54)	*phbA*$^+$, *phbB*$^-$, *phbC*$^+$, ORF2$^+$	LB-Tc-Glu	4.7	520	<20	<20	2.9

ORF, open-reading frame

[a] The cells were grown in media that promoted PHB accumulation (see Materials and methods), and were harvested after 36 h of cultivation. The media were supplemented with acetate (20 mM) for *T. violaceae*, with fructose (0.5%, w/v) for *A. eutrophus*, and with glucose (0.5%, w/v) for *E. coli*. The activities of PHA synthase were measured in crude cellular extracts by a slightly modified radiometric assay with [3-^{14}C]-D-(−)-3-hydroxybutyryl-CoA as described recently (Steinbüchel et al. 1992). The activities of β-ketothiolase and NADH- or NADPH-dependent acetoacetyl-CoA reductase were determined in soluble protein fractions of the cells by spectroscopic assays according to the methods described by Nishimura et al. (1978) and Lynen and Wieland (1955). Crude cellular extracts and the soluble protein fractions from the cells were obtained as described by Schubert et al. (1988). Soluble protein was determined as described by Lowry et al. (1951)

[b] One unit of enzyme activity is defined as the amount required to transform 1 μmol of substrate per minute

pHP1016::E54 was mobilized from *E. coli* S17-1 to various PHA-negative mutants and PHA-leaky mutants of *A. eutrophus*. In transconjugants of PHB$^-$4, for example, the ability to synthesize PHA was restored, as indicated by increased opacity of the colonies appearing on MM agar plates containing 0.005% (w/v) ammonium chloride and 0.5% (w/v) fructose. If the cells were analysed for the presence of enzyme activities relevant for the biosynthesis of PHA, it became obvious that E54 conferred the ability to synthesize PHA synthase to PHB$^-$4 (Table 1). In liquid culture with fructose as sole carbon source and under nitrogen-limiting conditions PHB occurred up to 24.8% of the cellular dry matter. If 0.5% (w/v) sodium gluconate or the sodium salts of propionate, valerate, heptanoate, or octanoate (each at a concentration of 0.1%, w/v) were used as carbon source, the cells contained PHA at fractions of 24.4, 8.0, 10.7, 12.4 and 22.1%, respectively, of the cellular dry matter. In PHA derived from cells grown with propionate, valerate or heptanoate, 3-hydroxyvaleric acid occurred at molar fractions of 2.1, 10.4 and 8.6%, respectively, in addition to 3-hydroxybutyric acid (3HB). The PHA of octanoate-grown cells contained approximately 1 mol% 3-hydroxyhexanoate in addition to 3HB. In transconjugants of PHA-leaky mutants, e.g. H1479, the accumulation of PHB was not significantly affected by the presence of pHP1016::E54.

Recombinant cells of *E. coli* harbouring pHP1016::E54 accumulated a small amount of PHB (2.9% of the cellular dry matter) after cultivation in LB medium containing 0.5% (w/v) glucose. Only PHA synthase and β-ketothiolase were detected at appreciable activities in strains harbouring pHP1016::E54, whereas activities of NADH- or NADPH-dependent acetoacetyl-CoA reductase were not detectable (Table 1).

Optical properties of PHB

The specific optical rotation ($[\alpha]_{589}^{20}$) of the methyl 3-hydroxybutyric acids liberated by methanolysis of PHB accumulated by cells of *T. violaceae* or of *A. eutrophus* PHB$^-$4, which harboured pHP1016::E54, was negative. These values were similar to those obtained for the methyl esters derived from PHB isolated from *A. eutrophus* H16 and suggested that the polyesters synthesized in the wild-type as well as in the recombinant strain consist of the (*R*)-enantiomer rather than of the (L)-enantiomer.

Determination of the nucleotide sequence

After cloning of fragment E54 into the Bluescript vector SK$^-$, the nucleotide sequence of E54 was obtained from overlapping partial sequences determined for both strands by applying the primer hopping strategy and by subjecting hybrid plasmids of Bluescript vector SK$^-$ to the dideoxy-chain-termination method. Universal and synthetic oligonucleotides were used as primers (Fig. 1). Because the nucleotide sequence analysis (see below) of E54 indicated that the region relevant for PHA biosynthesis was not complete, we determined also the nucleotide sequence of the adjacent fragment E20. Several

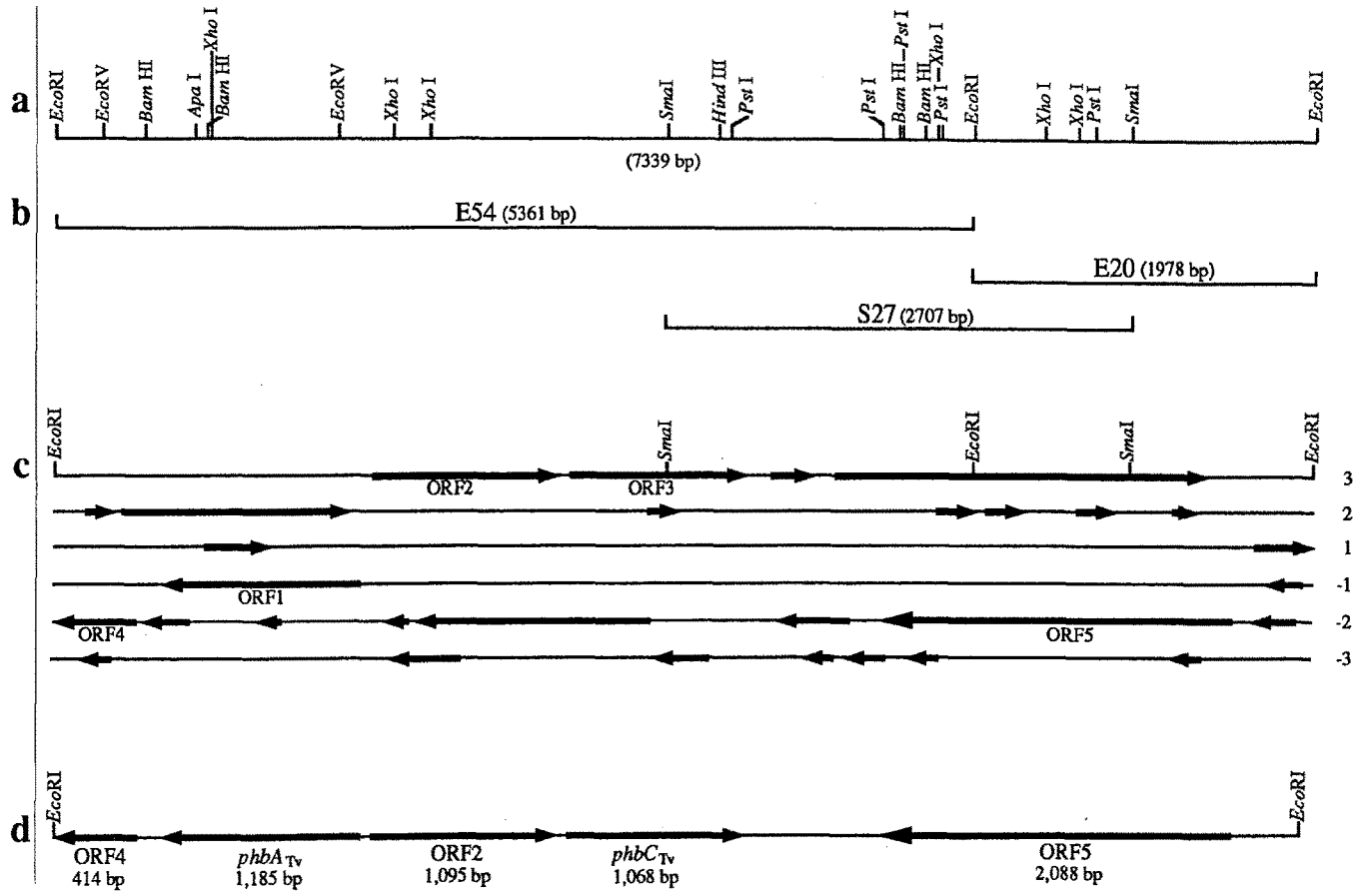


Fig. 1a–d. Molecular organization of the *Thiocystis violaceae* PHA-locus, restriction endonuclease sites, and DNA sequencing strategy. **a** Physical map as determined by restriction analysis. **b** Subfragments relevant for nucleotide sequence analysis and for heterologous expression studies. **c** Positions and orientations of open reading frames (ORFs) with more than 150 nucleotides. The numbers indicate those five ORFs that probably represent coding DNA. **d** Organization of $phbA_{Tv}$, $phbC_{Tv}$, ORF2, ORF4, and ORF5

ORFs were identified in the 7339-bp region; however, probably only five represented coding regions according to the rules of Bibb et al. (1984, see below). The nucleotide sequence of the overlapping region of the 2.7-kbp *Sma*I fragment (S27, Fig. 1), which was obtained from the hybrid phage harbouring the three *Eco*RI-restriction fragments mentioned above, demonstrated that E54 and E20 are directly linked and confirmed the results of the hybridization experiments.

Codon usage

For E54 plus E20 a G+C content of 63.7 mol% was determined, which is close to the G+C content determined for the total genomic DNA of this strain (63.1 mol%, Mandel et al. 1971). A highly biased choice for codons with either a G or a C at the third position (Table 2) occurred for ORF1 (83.3 mol%), ORF2 (90.0), ORF3 (90.1), ORF4 (79.1), and ORF5 (82.8); according to Bibb et al. (1984), the theoretical value is 84.5 mol%. In contrast, the G+C contents at the second position were 48.7 (ORF1), 45.9 (ORF2), 34.1 (ORF3), 34.5 (ORF4), and 42.5 mol% (ORF5) (46.0 mol% theoretical value); and the G+C contents at the first position were 64.7 (ORF1), 55.5 (ORF2), 57.2 (ORF3), 54.0 (ORF4), and 67.1 mol% (ORF5) (67.0 mol% theoretical value).

Properties of the putative ORF2 and ORF3 translation products

ORF2 (1095 bp) and ORF3 (1068 bp) were separated by an intergenic region of 63 bp. As both ORFs were preceded by tentative ribosomal binding sites, the ATG codons at positions 1869 and 3028 in Fig. 2 represent the putative translational start codons of ORF2 and ORF3, respectively. When the amino acid sequence deduced from both ORFs was compared with the primary structures of other proteins, the most extended homologies were obtained with the putative gene products of ORF2 and $phbC_{Cv}$ from a similar region of the *C. vinosum* genome (Figs. 3 and 4), which was analysed recently (Liebergesell and Steinbüchel 1992). Whereas the nucleotide sequence identity amounted to 82.5 and 77.2%, amino acid identities of 74.7 and 87.6% were revealed for ORF2 and ORF3, respectively. The putative ORF3 translation product exhibited only weak homology with the synthase of *A. eutrophus* H16 (21.8% amino acid identity) or with either synthase of *P. oleovorans* (26.3

Table 2. Codon usage in *phbA*, *phbC*, open-reading frame ORF2, ORF4, and ORF5

Amino acid	Usage in gene					
	Codon	$phbA_{Tv}$	$phbC_{Tv}$	ORF2	ORF4	ORF5
Ala	GCU	4	0	0	1	2
	GCA	2	4	5	0	4
	GCC	26	14	19	5	34
	GCG	26	4	12	2	45
Arg	AGA	0	0	0	0	1
	CGA	0	0	1	0	2
	AGG	1	1	1	0	6
	CGU	4	0	0	1	9
	CGG	2	3	2	1	7
	CGC	9	5	20	7	34
Asn	AAU	5	2	1	2	6
	AAC	5	16	14	4	7
Asp	GAU	11	2	3	6	12
	GAC	10	32	20	5	25
Cys	UGU	2	0	0	0	3
	UGC	4	4	2	1	2
Gln	CAA	3	0	5	3	5
	CAG	17	15	17	5	34
Glu	GAA	4	6	5	4	8
	GAG	15	8	19	7	37
Gly	GGA	5	3	0	2	2
	GGU	9	0	1	1	9
	GGG	5	0	2	1	10
	GCG	24	23	18	2	24
His	CAU	1	1	2	0	5
	CAC	4	3	3	0	6
Ile	AUA	0	0	0	0	1
	AUU	1	1	1	1	6
	AUC	22	19	2	9	32
Leu	UUG	3	2	2	0	11
	UUA	0	0	0	0	0
	CUU	1	2	1	2	6
	CUA	0	0	0	0	0
	CUC	12	15	13	8	29
	CUG	13	19	12	3	40
Lys	AAA	0	4	3	2	5
	AAG	18	19	17	5	18
Met	AUG	16	10	26	9	22
Phe	UUU	3	0	1	0	1
	UUC	7	11	11	7	22
Pro	CCA	0	1	1	0	4
	CCU	1	1	1	0	1
	CCG	13	10	8	1	13
	CCC	4	9	6	2	9
Ser	AGU	2	0	0	1	2
	UCU	1	0	0	0	1
	UCA	0	0	1	0	1
	UCC	5	3	7	2	9
	UCG	6	4	3	2	12
	AGC	6	9	12	4	13
Thr	ACA	0	0	2	1	1
	ACU	2	1	0	0	0
	ACG	6	4	8	4	16
	ACC	18	16	23	6	12
Trp	UGG	4	6	11	1	6
Tyr	UAU	1	6	2	2	4
	UAC	1	11	7	3	8
Val	GUA	1	0	0	0	0
	GUU	3	1	0	1	4
	GUC	15	18	5	3	30
	GUG	11	7	2	2	14

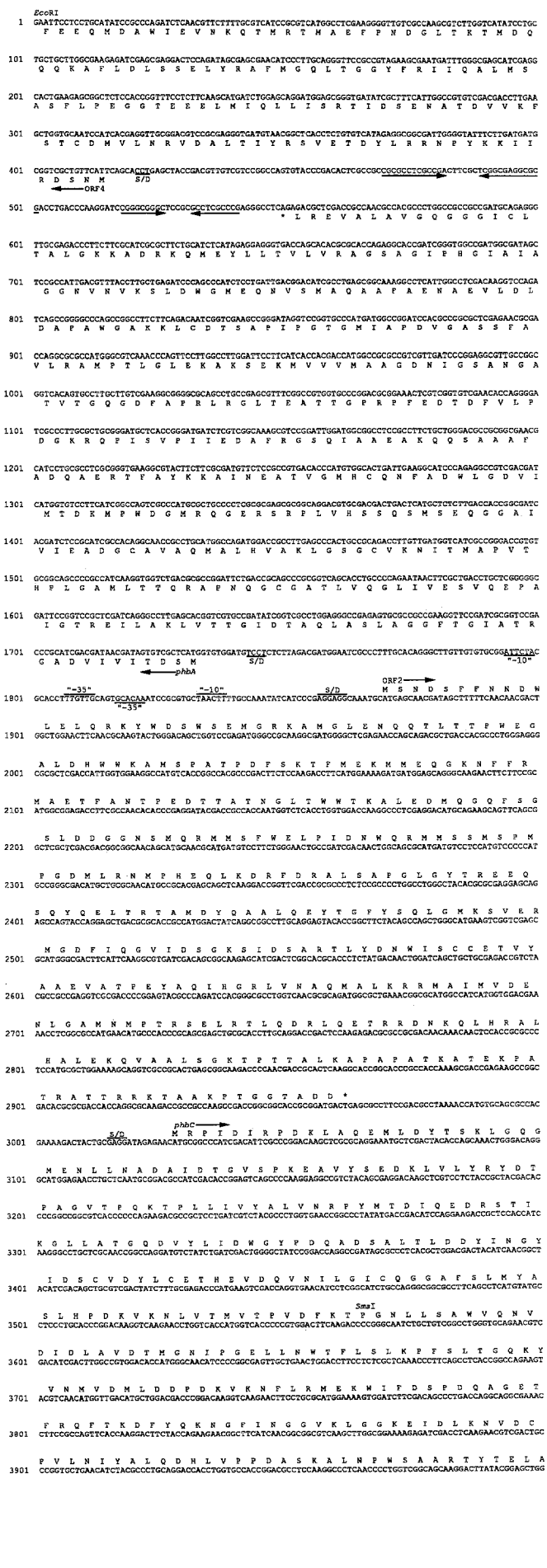
and 21.0%). Therefore, ORF3 probably encodes for the low relative molecular mass type PHA synthase (M_r 39550), which was recently detected in *C. vinosum* strain D. The function of the gene product of ORF2 (M_r 41450) is not known as is the function of the corresponding gene in *C. vinosum*.

At a distance of 31 bp upstream of ORF2, a region was identified (TTGTTG-20 bp-TAACTT) that was similar to the *E. coli* consensus sequence for σ^{70} dependent promoters (TTGACA-15/21 bp-TATAAT; Hawley and McClure 1983) and to the ORF2 promoter in *C. vinosum* (TTGTTG-15 bp-CATGAT). Downstream of ORF3 we identified an inverted repeat, which may represent a factor-dependent transcriptional terminator (Fig. 2). According to Tinoco et al. (1973), the free energy of this structure is approximately −302 KJ/mol.

Properties of the putative ORF1 translation product

ORF1 (1185 bp) is preceded by a tentative ribosomal binding site. Analysis of the amino acid sequence deduced from ORF1 revealed significant homologies with the primary structures of β-ketothiolase of *C. vinosum* (87.3% amino acid identity; Fig. 5), *A. eutrophus* (68.5%), *Z. ramigera* (58.8%), *E. coli* (44.3%), *Saccharomyces uvarum* (40.9%), and of *Rattus norvegicus* (42.5%). It was concluded that ORF1 represents the structural gene for the biosynthetic β-ketothiolase ($phbA_{Tv}$) of *T. violacea* with a predicted M_r of 40850. The nucleotide sequences of $phbA_{Tv}$ and $phbC_{Cv}$ exhibited 76.1% identity.

$PhbA_{Tv}$ was separated by 137 bp from ORF2, and the gene was oriented opposite to ORF2 and ORF3. Approximately 61 bp upstream of $phbA_{Tv}$ a region was identified (TTGTGC-17 bp-TAGAAT) the sequence of which revealed significant homology to the *E. coli* consensus sequence for a σ^{70}-dependent promoters and was identical with the $phbA_{Cv}$ promoter. The −35 region of this putative $phbA_{Tv}$ promoter was overlapping with the −35 region of the putative ORF2 promoter, as in *C. vinosum*. Downstream of $phbA_{Tv}$ an inverted repeat exhibiting a free energy of −163 KJ/mol (Tinoco et al. 1973) was detected, which may represent a factor-dependent transcriptional terminator (Fig. 2).



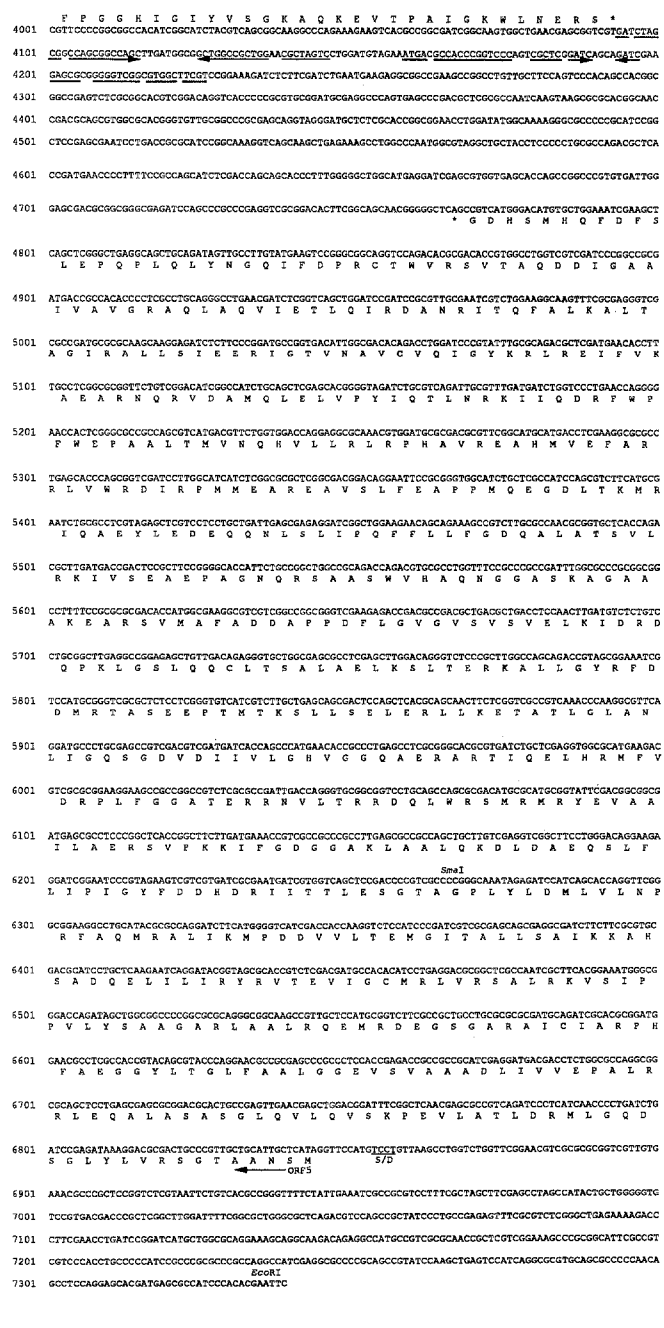


Fig. 2. Nucleotide sequence of the region of the *Chromatium vinosum* genome relevant for PHA synthesis. Amino acids deduced from the nucleotide sequence of the tentative genes are specified by standard *one-letter abbreviations.* The putative ribosome binding sites (Shine-Dalgarno, S/D) are *boxed.* The −10 and the −35 regions of the putative promoters of *β*-ketothiolase and of ORF2 are *underlined.* The positions of hairpin-like structures downstream of $phbC_{Tv}$ and $phbA_{Tv}$ as shown by the software "Fold" of the GCG-package are indicated by *opposed arrows*

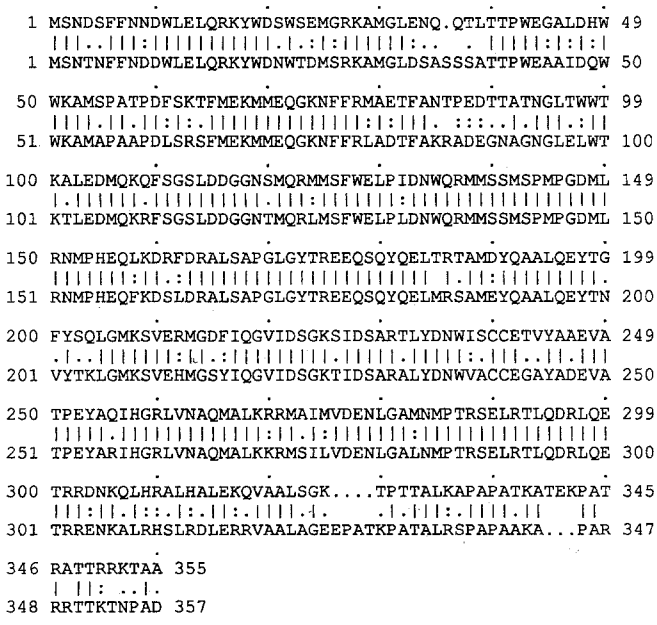


Fig. 3. Alignment of the amino acid sequences of ORF2 gene products deduced from the nucleotide sequence of ORF2 of *T. violaceae* (*upper line*) and of ORF2 of *C. vinosum* (*bottom line*, Liebergesell and Steinbüchel 1992). Amino acids are specified by standard *one-letter abbreviations*, and the *numbers* indicate the position of the amino acid within the respective proteins

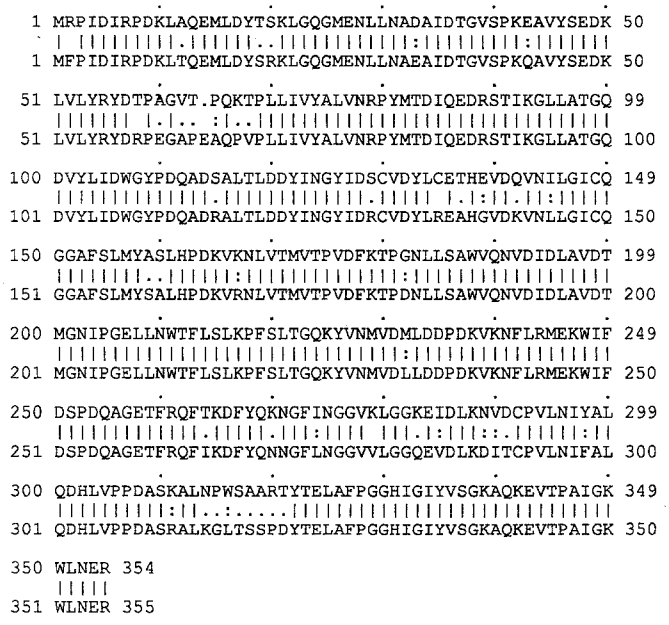


Fig. 4. Alignment of the amino acid sequences of PHA synthases deduced from the nucleotide sequences of $phbC_{Tv}$ (*upper line*) and of $phbC_{Cv}$ (*bottom line*, Liebergesell and Steinbüchel 1992). Amino acids are specified by standard *one-letter abbreviations*, and the *numbers* indicate the position of the amino acid within the respective proteins

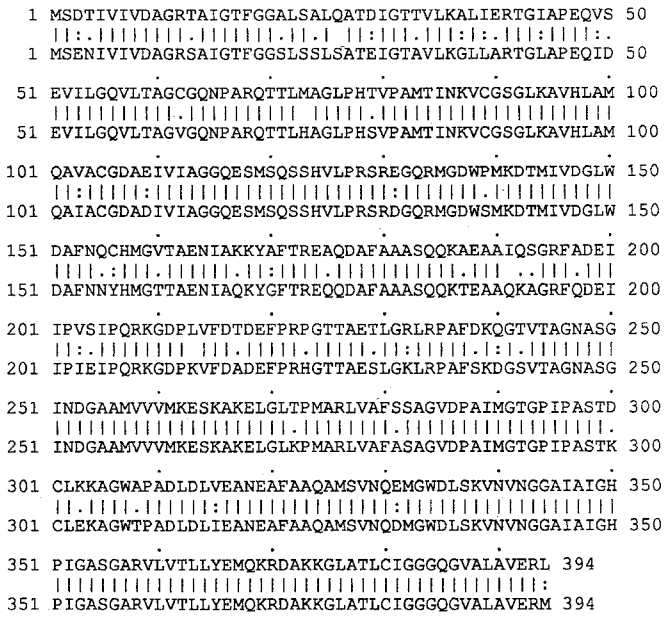


Fig. 5. Alignment of the amino acid sequences of β-ketothiolase deduced from the nucleotide sequences of $phbA_{Tv}$ (*upper line*) and of $phbA_{Cv}$ (*bottom line*, Liebergesell and Steinbüchel 1992). Amino acids are specified by *standard one-letter abbreviations*, and the *numbers* indicate the position of the amino acid within the respective proteins

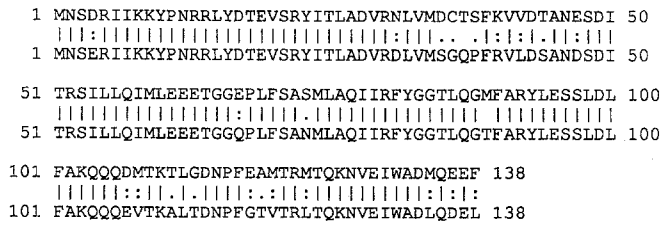


Fig. 6. Alignment of the amino acid sequences of ORF4 gene products deduced from the nucleotide sequences of ORF4 of *T. violaceae* (*upper line*) and of ORF4 of *C. vinosum* (*bottom line*, Liebergesell and Steinbüchel 1992). Amino acids are specified by standard *one-letter abbreviations*, and the *numbers* indicate the position of the amino acid within the respective proteins

Properties of the putative ORF4 translation product

An ORF was identified 133 bp downstream from $phbA_{Tv}$, which was referred to as ORF4 and which exhibited 79.1% nucleotide sequence and 82.6% amino acid identity with the sequence of ORF4, which was identified in the corresponding region of the *C. vinosum* genome (Fig. 6), or of the putative translational product, respectively (Liebergesell and Steinbüchel 1992). ORF4 was preceded by a tentative ribosome binding site. ORF4 was not completed since the *Eco*RI-restriction site was located in the 3′-region of the gene.

Properties of the putative ORF5 translation product

ORF5 extended from the ATG-codon at position 6852 to position 4764. However, considering the rules affecting m-RNA translatability (DeBoer and Hui 1990), it is more likely that only the second ATG-codon at position

6843, which is preceded by a reliable Shine-Dalgarno (S/D) sequence, is the translation start codon thus resulting in a translation product of M_r 76428. The amino acid sequence deduced from the nucleotide sequence exhibited no homologies to proteins available from data banks.

Discussion

In this study the *T. violaceae* PHA synthase and β-ketothiolase structural genes have been cloned together with two other genes presumably relevant for PHA synthesis in this bacterium. A fifth gene was identified upstream and antilinear to PHA synthase. To the authors knowledge, this is the first report on genes that have been cloned and sequenced from this anoxygenic photosynthetic purple sulphur bacterium. The genes investigated in this study as well as the organization of the genes exhibited striking homologies to the corresponding region of *C. vinosum*, which was analysed recently (Liebergesell and Steinbüchel 1992). However, in contrast to the fragment obtained from *C. vinosum*, the fragment analysed in this study obviously did not harbour the structural gene for a pyridine-nucleotide-dependent acetoacetyl-CoA reductase.

PHA synthase and β-ketothiolase were not only heterologously expressed in *A. eutrophus*; they also constituted a functionally active PHA-biosynthetic pathway in PHA-negative mutants of *A. eutrophus*, although a gene for acetoacetyl-CoA reductase was absent. Since the ability of a PHA-negative mutant of *A. eutrophus* to accumulate PHB was also regained with genomic fragments of *Rhodococcus ruber* NCIMB40126, which obviously encoded only for PHA synthase (Pieper and Steinbüchel 1992), this confirmed the recent assumption (Steinbüchel and Schlegel 1991) that the NADPH-dependent acetoacetyl-CoA reductase encoded by *phbB* is not essentially required for PHA biosynthesis in *A. eutrophus*. Either an as yet undetected isoenzyme of the NADPH-dependent reductase may exist in *A. eutrophus*, or – more likely – a route exists in *A. eutrophus* that circumvents the reaction catalysed by the *phbB* gene product. As *A. eutrophus* possesses an NADH-dependent reductase leading to the formation of L(+)-3-hydroxybutyryl-CoA, which in reconstituted enzyme systems did not contribute to synthesis of PHB (Haywood et al. 1988), two enoyl-CoA hydratases may catalyse the conversion of the L(+)- to the D(−) isomer of 3-hydroxybutyryl-CoA. These studies might therefore indicate that the *R. rubrum* PHA-biosynthetic pathway may be present in *A. eutrophus* as an alternative pathway in addition to its own pathway.

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