Multiple evidence for widespread and general occurrence of type-III PHA synthases in cyanobacteria and molecular characterization of the PHA synthases from two thermophilic cyanobacteria: *Chlorogloeopsis fritschii* PCC 6912 and *Synechococcus* sp. strain MA19

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Eleven different cyanobacteria were investigated with respect to their capabilities to synthesize poly-3-hydroxybutyrate [poly(3HB)] and the type of poly- β -hydroxyalkanoic acid (PHA) synthase accounting for the synthesis of this polyester. Several methods, including (i) Southern blot analysis using a *phaC*-specific DNA probe, (ii) Western blot analysis using specific polyclonal anti-PhaE antibodies raised in this study against PhaE of *Synechocystis* sp. strain PCC 6803, (iii) generation and sequence analysis of PCR products using *phaC*-specific oligonucleotides as primers, and/or (iv) cloning and sequence analysis of PHA synthase structural genes, were used to provide evidence for the presence of a type-III PHA synthase in the following cyanobacteria: *Synechococcus* sp. strains MA19 and PCC 6715, *Chlorogloeopsis fritschii* PCC 6912, *Anabaena cylindrica* SAG 1403-2, *Cyanothece* sp. strains PCC 7424, PCC 8303 and PCC 8801, and *Gloeocapsa* sp. strain PCC 7428. The screening was compared with corresponding studies using crude protein extracts and genomic DNA of *Synechocystis* sp. strain PCC 6803, as a positive control, which is so far the only cyanobacterium for which molecular data of the PHA synthase genes are available. No evidence for the presence of a type-III PHA synthase could be obtained for only three of the eleven investigated cyanobacteria (*Stanieria* sp. strain PCC 7437, *Cyanothece* sp. strain PCC 8955 and *Gloeotheca* sp. strain PCC 6501). The entire PHA synthase structural genes of the two thermophilic cyanobacteria *Synechococcus* sp. strain MA19 and *Chlorogloeopsis fritschii* PCC 6912, and in addition a central region of the *phaC* gene of *Cyanothece* sp. strain PCC 8303, were cloned, sequenced and also heterologously expressed in *Escherichia coli*.

Keywords: inverse PCR, thermotolerant enzymes, PHB, bioplastic, *Anabaena cylindrica*, *Gloeotheca* sp.

INTRODUCTION

Cyanobacteria are oxygenic, photoautotrophic prokaryotes which possess two photosystems (PSI and PSII)

Abbreviations: CDM, cell dry matter; PHA; poly(3-hydroxyalkanoate); PHB, poly(3-hydroxybutyrate); 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvaleric acid.

The NCBI accession numbers for the *phaC* gene sequences reported in this paper are AY030295 (*phaC*_{MA19}) and AF371369 (*phaC*_{Cf}).

releasing electrons from water and which fix carbon dioxide via the Calvin–Benson–Bassham pathway. The fixed carbon is partially deposited in the form of intracellular polymers such as glycogen, other polyglucans and cyanophycin, which serve as carbon and energy reserves. The presence of poly(3-hydroxyalkanoate) (PHA) in about 50 strains of four different phylogenetic subsections of cyanobacteria has been reviewed by Vincenzini & De Philippis (1999) (and literature cited therein). However, detailed studies on

biosynthesis and accumulation of PHA have so far only been done for a few species of cyanobacteria, in particular for *Chlorogloea fritschii* (Carr, 1966; Jensen & Sicko, 1971), *Gloeocapsa* sp. (Rippka *et al.*, 1971), *Oscillatoria limosa* (Stal *et al.*, 1990), *Gloeotheca* sp. PCC 6909 (Arino *et al.*, 1995), some species of the genus *Spirulina* (Vincenzini *et al.*, 1990; De Philippis *et al.*, 1992) and *Synechococcus* sp. strain MA19 (Miyake *et al.*, 1996). So far, only 3-hydroxybutyrate (3HB) and 3-hydroxyvaleric acid (3HV) from the various known constituents of PHAs (Steinbüchel & Valentin, 1995; Steinbüchel, 2001) have been identified in the PHAs accumulated by cyanobacteria (Stal *et al.*, 1990; De Philippis *et al.*, 1992; Hein *et al.*, 1998). The occurrence of the copolyester poly(3HB-co-3HV) was observed in *Anabaena cylindrica* 10C (Lama *et al.*, 1996).

The biochemical and molecular basis of PHA synthesis has been investigated intensively in many micro-organisms (Steinbüchel & Hein, 2001). However, in cyanobacteria, studies at the molecular level regarding PHA biosynthesis have been reported only for *Synechocystis* sp. strain PCC 6803 (Hein *et al.*, 1998; Taroncher-Oldenburg *et al.*, 2000). Only from this cyanobacterium has the PHA synthase gene been cloned (Hein *et al.*, 1998). Interestingly, the PHA synthase of *Synechocystis* sp. PCC 6803 is composed of two different subunits, encoded by two contiguous, adjacent co-transcribed genes, referred to as *phaE* and *phaC* (Hein *et al.*, 1998). The translational products of these two structural genes showed similarity to the corresponding PHA synthases of the anoxygenic purple sulfur bacteria *Allochrochromatium vinosum* (Liebergesell & Steinbüchel, 1992; Liebergesell *et al.*, 1994), *Thiocystis violacea* (Liebergesell & Steinbüchel, 1993), *Thiococcus* (formerly *Thiocapsa*) *pfennigii* (Liebergesell *et al.*, 2000) and *Ectothiorhodospira shaposhnikovii* (Genbank accession no. AAG30259 for *phaC* and AAG30260 for *phaE*). These enzymes belong to the type-III PHA synthases characteristic of γ -Proteobacteria (Rehm & Steinbüchel, 1999; Steinbüchel & Hein, 2001). Expression of functionally active type-III PHA synthases requires the expression of both subunits PhaE and PhaC; PhaE alone was completely inactive and PhaC alone exhibited only negligible activity, if at all (Liebergesell *et al.*, 1994; Müh *et al.*, 1999; Jia *et al.*, 2000). The PhaC proteins exhibit much higher similarities than the PhaE proteins and the latter revealed absolutely no similarities to other PHA synthases, but contained two amino acid stretches at the C-terminal regions like PHA granule binding proteins (phasins), which might serve as binding domains of PHA synthases to the surface of PHA granules (Liebergesell *et al.*, 2000). However, the function of PhaE has not yet been revealed.

Due to knowledge of only one cyanobacterial PHA synthase, it is still unclear whether the type-III PHA synthase is a unique enzyme of cyanobacteria, or whether other types (Rehm & Steinbüchel, 1999) also occur in this group of photosynthetic bacteria. The aim of this study was to investigate the distribution of type-III PHA synthases among cyanobacteria and to clone the

pha genes from two thermophilic cyanobacteria. Thermotolerant PHA synthases have so far never been described and corresponding genes are not available. Such enzymes might be useful for biotechnological applications such as *in vitro* PHA biosynthesis processes (Steinbüchel, 2001).

METHODS

Bacterial strains and plasmids. An axenic culture of *Synechococcus* sp. MA19, which was also used in a previous study (Hai *et al.*, 1999), was a gift of the culture collection of the Molecular Bioenergetics Laboratory at the National Institute of Bioscience and Human-Technology (Tzukuba, Ibaraki, Japan). *Anabaena cylindrica* SAG 1403-2 was obtained from the Sammlung von Algenkulturen Göttingen (Göttingen, Germany), whereas *Chlorogloeopsis fritschii* PCC 6912 and other axenic cyanobacteria used in this study were provided by Dr Rippka from the Pasteur Culture Collection (Institut Pasteur, Paris, France). The cyanobacteria, other bacterial strains and plasmids used in this study are listed in Table 1.

Media and cultivation conditions. Thermophilic and mesophilic cyanobacteria were grown photoautotrophically at 50 and 28 °C, respectively, in liquid BG11 medium (Rippka *et al.*, 1979) with irradiation ($100 \mu\text{E s}^{-1} \text{m}^{-2}$) and shaking (100 r.p.m.). To study PHA accumulation in cyanobacteria, two-stage cultivation experiments were done. The cultures were first grown in 500 ml flasks containing 200 ml full strength BG11 medium at 28 or 50 °C with shaking under irradiation applying a photon flux of approximately $150 \mu\text{E m}^{-2} \text{s}^{-1}$. Since the doubling times of the strains studied differed, the second stage was carried out as follows. After 4 or 7 d, when the cultures were in exponential growth phase, the medium was removed by centrifugation, the cell pellets were washed with sterile nitrogen-free medium (the sodium nitrate component was omitted; BG11o) and the cells were then resuspended in 50 ml sterile BG11o medium containing 15 mM acetate. PHA accumulation was promoted by incubating the cells at low irradiation (approx. $50 \mu\text{E m}^{-2} \text{s}^{-1}$) for a further 7 d with shaking. The medium volume was adjusted daily with sterile double-distilled water during cultivation.

Triclosan ($1\text{--}5 \mu\text{g ml}^{-1}$) was added to the second phase when the cells had been transferred into BG11o medium plus 15 mM acetate. For studying the influence of triclosan on PHA accumulation, a concentration of $1.5 \mu\text{g ml}^{-1}$ was applied for all strains and growth was monitored for a further 7 d.

Escherichia coli strains were grown with shaking (150 r.p.m.) at 37 °C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) with or without antibiotics. Competent cells of *Escherichia coli* were prepared by using the standard CaCl_2 method (Sambrook *et al.*, 1989). For recombinant strains of *Escherichia coli* harbouring *pha* genes, PHA accumulation experiments were carried out in liquid LB containing 0.6% (w/v) glucose as carbon source. In addition, 50 μM thiamine and 0.2 mM IPTG were added if *Escherichia coli* harboured pBluescriptSK⁻ (pSK⁻) or derivatives of this vector.

Preparation of PHA granules. PHA granules were isolated from acetate-grown cells of *Synechocystis* sp. PCC 6803 by centrifugation in a glycerol gradient as described previously (Hein *et al.*, 1998). The PHA granules were enriched in the 65% (v/v) glycerol fraction, which was confirmed by GC

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype characteristics*	Reference or source†
Cyanobacteria		
<i>Anabaena cylindrica</i> SAG 1403-2	Growth at 28 °C	SAG
<i>Chlorogloeopsis fritschii</i> PCC 6912	Optimal growth at 50 °C	PCC
<i>Cyanothece</i> sp. strain PCC 7424	Growth at 28 °C	PCC
<i>Cyanothece</i> sp. strain PCC 8303	Growth at 28 °C	PCC
<i>Cyanothece</i> sp. strain PCC 8801	Growth at 28 °C	PCC
<i>Cyanothece</i> sp. strain PCC 8955	Optimal growth at 28 °C	PCC
<i>Gloeocapsa</i> sp. strain PCC 7428	Growth at 28 °C	PCC
<i>Gloeotheca</i> sp. strain PCC 6501	Growth at 28 °C	PCC
<i>Stanieria</i> sp. strain PCC 7437	Growth at 28 °C	PCC
<i>Synechococcus</i> sp. strain MA19	Optimal growth at 50 °C	Miyake <i>et al.</i> (1996)
<i>Synechococcus</i> sp. strain PCC 6715	Optimal growth at 50 °C	PCC
<i>Synechocystis</i> sp. strain PCC 6803	Growth at 28 °C	PCC
<i>Escherichia coli</i>		
TOP10	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δlac U169</i> (ϕ 80 <i>lacZ</i> DM15)	Invitrogen
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^q</i> ZDM15, Tn10(Tet ^r)]	Stratagene
S17-1	<i>thi1 proA hsdR17 hsdM⁺ recA</i> RP4- <i>tra</i> -function	Simon <i>et al.</i> (1983)
Plasmids		
pSK ⁻	<i>amp lacZp/o</i> , T7 & T3 promoters	Stratagene
pMa/c5-914	<i>amp cat c1857ts lacZp/o</i> , TIR, P _L & P _R	SK 6772
pMa/c5-914::EC1.1	1.1 kbp <i>EcoRI</i> – <i>Clai</i> fragment of <i>Synechocystis</i> sp. PCC 6803 harbouring His ₆ -tagged <i>phaE</i> _{Syn} in pMa/c5-914	This study
pSK:: <i>phaC</i> _{MA19}	1.3 kbp PCR product of <i>PhaC</i> _{MA19} inserted in pSK ⁻	This study
pSK:: <i>phaC</i> _{Cr}	1.32 kbp PCR product of <i>PhaC</i> _{MA19} inserted in pSK ⁻	This study
pSKAB _{Syn}	pSK ⁻ harbouring <i>phaA</i> and <i>phaB</i> (<i>phaA</i> - <i>B</i> _{Syn}) from <i>Synechocystis</i> sp. PCC 6803	This study
pSKABC _{Cr}	3.6 kbp harbouring <i>phaA</i> - <i>B</i> _{Syn} and <i>phaC</i> _{Cr} from <i>Chlorogloeopsis fritschii</i> PCC 6912	This study
pSKABC _{MA}	3.6 kbp harbouring <i>phaA</i> - <i>B</i> _{Syn} and <i>phaC</i> _{MA19} from <i>Synechococcus</i> sp. MA19	This study
pSKABEC _{Syn}	pSK ⁻ harbouring <i>phaA</i> , <i>phaB</i> , <i>phaE</i> and <i>phaC</i> from <i>Synechocystis</i> sp. PCC 6803	This study

* Mesophilic and thermophilic cyanobacteria were cultivated at 28 and 50 °C, respectively (the optimal temperature for growth was used).

† PCC, Pasteur Culture Collection, Institut Pasteur, Paris, France; SAG, Sammlung von Algenkulturen Göttingen, Göttingen, Germany; SK, Culture Collection of the Institute of Microbiology, Münster, Germany.

analysis. The granule-bound proteins were analysed by Western immunoblotting.

DNA isolation and manipulation. Total DNA from the various cyanobacterial strains was extracted following a previously described protocol (Hein *et al.*, 1998). Plasmid isolations were done by standard methods (Sambrook *et al.*, 1989) or by using commercial kits (Qiagen). Restriction and ligation of DNA molecules were done according to standard protocols (Sambrook *et al.*, 1989) or according to the instructions of the suppliers of the enzymes.

Southern hybridization experiments. The *phaC*_{Syn} gene of *Synechocystis* sp. PCC 6803 was amplified by PCR, employing the primers Haphapcr1 and Haphapcr2 (Table 2) and *Vent* polymerase (New England Biolabs), according to the Biochemica PCR applications manual (1995). The PCR products (approx. 560 bp) were purified by using the Nucleotrap kit

(Macherey & Nagel), following the instructions of the manufacturer, and were labelled by using the DIG-High Prime kit (Boehringer Mannheim). The hybridization, which was done at 53 °C, and other related procedures followed standard methods (Sambrook *et al.*, 1989). For visualizing the chemoluminescence, the substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decal}12-4-yl) phenyl phosphate (CSPD) was used.

Cloning of *phaE*_{Syn} and purification of His₆-tagged *PhaE*_{Syn} from recombinant *Escherichia coli*. For cloning of *phaE*_{Syn} into *Escherichia coli*, PCR was done by using His₆*phaE*_{Syn} as sense and *PhaE*reverse as reverse primers (Table 2), which were deduced from the upstream and downstream regions, respectively, of *slr1829* of *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996). The *phaE*_{Syn}-specific PCR product obtained was purified and ligated into pMa/c5-914 (Table 1), which harbours, besides other genes, the *c1857ts* gene encoding the

Table 2. Primers used in this study

PCR primers for the cloning of His₆-tagged *phaE*_{Syn} with inserted restriction sites for *Nde*I and *Eco*RI (shown in italics). For cloning *phaC* from *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912 the primers were deduced from the upstream and downstream regions of *phaC*, except for the nucleotides shown in italics, which were inserted to generate *Bam*HI and *Apa*I, and *Nde*I and *Bam*HI restriction sites for the genes of strains MA19 and PCC 6912, respectively. PhaA and PhaB were obtained by PCR using sense and reverse primers pSKABsense and pSKABreverse. All primers were synthesized by MWG Biotech.

Primer	Nucleotide sequence (5' → 3')	Application
HisphaE _{Syn}	CATATGCATCACCATCACCATCACATGGAATCGACA	Overexpression
PhaEreverse	CTTAAGTACCACTTAATTGATAGGTAGCTAGACTT	Overexpression
Haphapcr1	GTTTTAATCGATTACGCNYTNGTNAAAYMGNCCNTAYATG	<i>phaC</i> screening
Haphapcr2	CGGGACTATRAADATCCAYTTYTCCATNCKTAGAAAGTT	<i>phaC</i> screening
MAphaCL	AAGTTGCTGAACCTTTTTCGCGCATG	Inverse PCR
MAphaCR	TGTAGCCGTTAATGTAGTCATCTAGA	Inverse PCR
PhaC69R	GCAGTTGGGCATTCAGAATACCTG	Inverse PCR
PhaC69L	ATATCCAGGCCCAACTTGAGAT	Inverse PCR
MaphaCsense	CAACTCCGGATCCAACAGATGAAG	Cloning of <i>phaC</i>
MaphaCreverse	GTCTCGAAAGGGCCCAACACAATC	Cloning of <i>phaC</i>
CfHisphaCs	TCTCCATATGCATCACCATCACCATCACGGCTGGAAATTTA	Cloning of <i>phaC</i>
CfphaCreverse	ATTCATAGGATCCCAGTTAAAAATGCGCT	Cloning of <i>phaC</i>
PhaABsense	AGCTTGAAATTTCCATATGCCCGGC	Overexpression
PhaABreverse	TTAGGATCCGTGGGCCCTTTAC	Overexpression

Fig. 1. Construction of the overexpression plasmid pMa/c5-914::*phaE*. His₆-tagged *phaE*_{Syn} was inserted into vector pMa/c5-914 harbouring a temperature-sensitive λ repressor gene (c1857s) as well as two antibiotic resistance genes, *cat* and *amp*, the promoter/operator region (*lacZp/o*) and the translation initiation region (TIR). Specific primers for the amplification of *phaE*_{Syn} are shown. PhaE was overexpressed in *Escherichia coli* and was purified to electrophoretic homogeneity using a Ni-NTA matrix chromatography column, as described in Methods. fdT indicates the transcription terminator of phage fd.

temperature-sensitive λ repressor. The construction of the hybrid plasmid pMa/c5-914::*EC1.1* is shown in Fig. 1. The His₆-tagged protein, PhaE_{Syn}, was then expressed in *Escherichia coli* and was purified to electrophoretic homogeneity employing a Ni-NTA agarose-matrix purification kit following the instructions of the manufacturer (Qiagen).

Preparation of soluble protein cell fractions from recombinants *Escherichia coli*. The recombinant strains of *Escherichia coli* were grown with shaking in 200 ml LB medium plus ampicillin (final concentration 100 μ g ml⁻¹) to an OD₆₀₀ of 0.5–0.7 at 37 °C. Then IPTG (0.2 mM) was added and the cells were cultivated for a further 4 h. The washed cell pellets (1 g) were then resuspended in a buffer containing 20 mM potassium phosphate, 500 mM sodium chloride and 0.5 mM EDTA and disintegrated by two passes through a French pressure cell at 10000 p.s.i. (1 p.s.i. = 6.9 kPa). The

crude extract was obtained by centrifugation at 20000 g at 4 °C for 15 min and the protein concentration was adjusted to 1 mg ml⁻¹.

Production and purification of antibodies against PhaE_{Syn}. The His₆-tagged PhaE_{Syn} was isolated from an SDS-polyacrylamide gel and submitted to Eurogentec for antibody production. The latter was achieved by three subcutaneous injections into a rabbit (animal code SA 6918; Eurogentec) over a period of 3 months following standard procedures. The antibodies were purified from the crude serum by using FPLC on a Protein A-Sepharose CL-4B affinity column (Hjelm *et al.*, 1972).

Western blotting and immunodetection employing anti-PhaE_{Syn} antibodies. Crude extracts were obtained from cells of cyanobacteria and recombinant *Escherichia coli* as follows.

The washed cells (approx. 0.2 g wet wt) were dissolved in 1 ml 10 mM Tris/HCl buffer, pH 8.0, containing 5 mM DTT, and subsequently disintegrated by 1 min treatment with a Sonoplus GM200 sonifier (Bandelin Electronic). Three volumes of this crude protein solution were then mixed with 1 vol. SDS-additive solution consisting of 8% (w/v) SDS, 40% (w/v) glycerol, 20% (v/v) 2-mercaptoethanol and 0.004% (w/v) bromophenol blue. The proteins were denatured by incubating at 95 °C for 5 min. The proteins were then separated by SDS-PAGE in 11.5% (w/v) polyacrylamide gels as described by Laemmli (1970). The protein bands from one gel were stained for proteins with Serva Blue R (Weber & Osborn, 1969), whereas the proteins from a second gel were blotted onto a nitrocellulose BA83 membrane (Schleicher & Schüll) using a semi-dry transfer blotter (Bio-Rad) and applying a voltage of 24 V for 100 min. The membrane was then equilibrated with 10 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl and 0.05% (v/v) Tween 20 (TNT buffer) and blocked as described previously (Hein *et al.*, 1998). The immunological detection followed a standard method (Sambrook *et al.*, 1989) with the only modification that 5% (w/v) skimmed milk in TNT buffer was used as blocking agent. The purified antibodies, as eluted from the protein A Sepharose-4B column, were diluted 1:1000 and used as the primary antibody. A solution of an anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma), diluted 1:30000, was used as the secondary antibody. After removing the excess of the secondary antibody by washing the membrane three times with TNT buffer containing 0.1% (w/v) bovine albumin fraction V and 0.1% (v/v) Nonidet P40, the bound antibodies were visualized with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) with a commercially available detection kit (Sigma) following the instructions of the manufacturer.

PCR using degenerate primers. The nucleotide sequences of two primers, Haphapcr1 (sense) and Haphapcr2 (reverse), were deduced from highly conserved regions of superfamily *phaC* genes (Table 2), including *phaC* of the anoxygenic phototrophic bacteria *Allochrochromatium vinosum* and *Thiocystis violacea*, and the cyanobacterium *Synechocystis* sp. PCC 6803. The regions corresponded to amino acid positions 85–97 and 275–263, respectively, in PhaC of *Synechocystis* sp. PCC 6803.

PCR was performed with DNA isolated from various cyanobacteria by using *Vent* DNA polymerase and applying the following temperature programme: 1 cycle of 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 80 s. The PCR products were purified by using the Nucleotrap kit (Macherey Nagel) and were subsequently ligated to pSK⁺-DNA, which was linearized by treatment with *EcoRV*. The ligation products were then transformed into *Escherichia coli* following standard protocols (Sambrook *et al.*, 1989).

Inverse PCR to clone the 5' and 3' regions of *phaC* and *phaE* from *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912. To clone the 5' and 3' regions adjacent to the PCR products, inverse PCR (Triglia *et al.*, 1988) was applied. Primers MaphaCL and MaphaCR (Table 2), corresponding to base positions 735–759 and 385–360 in *phaC*_{MA19} (accession no. AY030295), respectively, were used for *Synechococcus* sp. MA19 DNA. Similarly, PhaC69R and PhaC69L (Table 2), corresponding to base positions 643–666 and 302–323 in *phaC*_{CF} (accession no. AF371369), respectively, were used for the *Chlorogloeopsis fritschii* PCC 6912 template. The sequences of these primers were deduced from those of the 5' and 3' regions of the corresponding PCR products. Genomic

DNA was first restricted with *Pst*I, cyclic products were then obtained by incubation with T4 DNA ligase and these were linearized by digestion with *Ssp*I. Using *Vent* polymerase, the following temperature programme was applied to both DNA templates: 1 cycle of 95 °C for 2 min, 32 cycles of successive programming of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 2 min. The PCR products were then precipitated by adding 2.5 vols ethanol, washed with 70% (v/v) ethanol, dried at room temperature and dissolved in 20 µl 5 mM Tris/HCl, pH 8.0, containing 0.5 mM EDTA. About 400 ng PCR products were ligated at room temperature overnight with 400 ng *EcoRV*-linearized pSK⁺-DNA and were then transformed into *Escherichia coli* XL-1 Blue (see Fig. 3).

Cloning of *phaC* from *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912. The *phaC* genes from both strains were amplified by PCR from genomic DNA following the procedures described above. The nucleotide sequences of the primers were deduced from the nucleotide sequences of regions located upstream and downstream of the corresponding *phaC* gene, but allowing the generation of *Bam*HI and *Apa*I, or *Nde*I and *Bam*HI restriction sites for the genes of strains MA19 (*phaC*_{MA19}) and PCC 6912 (*phaC*_{CF}), respectively. The primers MaphaCsense and MaphaCreverse were used for cloning *phaC*_{MA19}, whereas the primers CfHisphaCs with a His₆ tag and CfphaCreverse were applied to clone *phaC*_{CF} (Table 2).

Construction of hybrid plasmids. To study PHA synthase activity of PhaC subunits some hybrid plasmids were constructed. The expression vectors were derived from pSK⁺ as follows. (i) A PCR product containing *slr1993*, encoding β -ketothiolase (*phaA*_{syn}), together with *slr1994*, encoding acetoacetyl CoA reductase (*phaB*_{syn}), from *Synechocystis* sp. PCC 6803 was amplified by using primers PhaABsense and PhaABreverse, which included degenerate *Nde*I and *Bam*HI restriction sites, respectively (Table 2). The purified PCR product (2.45 kbp) was cloned to obtain pSKABSyn (see Fig. 7). After digestion with *Bam*HI, the purified *phaAB*_{syn} fragment was ligated to the *Bam*HI-treated PCR product of *phaC*_{MA19} giving the *phaABC* fragment. (ii) The purified *phaABC* was ligated to *EcoRV*-digested pSK⁺. The hybrid plasmid obtained, pSKABC_{MA19}, was transformed into *Escherichia coli* XL-1 Blue. Similarly, other hybrid plasmids were constructed, such as pSKABC_{CF} and pSKABEC_{syn} containing fragment *phaEC*_{syn}, which was obtained in a previous study (Hein *et al.*, 1998). The hybrid plasmid pSKABEC_{syn} was also transformed into *Escherichia coli* S17-1 and served in this study as a positive control.

DNA sequencing, sequence analysis and alignments. DNA sequences were determined employing a model 4000L DNA sequencer (LI-COR) and a thermosequencing fluorescence-labelled primer cycle sequencing kit (Amersham Life Science), according to the instructions of the respective manufacturer.

The nucleic acid sequences obtained from both strands were analysed with a computer program available from the Heidelberg Unix Sequence Analysis Resources (HUSAR, release 4.0). Sequence comparisons were performed by using the network service programs BLAST provided by the National Center for Biotechnology Information (NCBI). The sequences corresponding to C-terminal amino acids of PhaE and PhaC, as well as the 120 aa of regions surrounding the substrate-binding sites (Cys-149 in PhaC of *Allochrochromatium vinosum*) and the first 355 aa of PhaC, were aligned using the CLUSTAL W and also CLUSTAL X programs provided by the European Bioinformatics Institute. The phylogenetic distance

tree was reconstructed by using PROTDIST and PROTPARS of PHYLIP (Phylogeny Inference Package; Felsenstein, 1989), which are available on the internet (<http://www.es.embnnet.org/Sevices/>).

Electrophoresis of proteins and nucleic acids. SDS-PAGE of proteins was performed in 11.5% (w/v) gels according to Laemmli (1970). Molecular mass marker proteins were purchased from Bio-Rad. Protein staining was done with Serva Blue R. Protein concentrations were estimated following the method of Bradford (1976). For analysis of nucleic acids, electrophoresis was done in 0.8 and 1.2% (w/v) agarose gels according to standard methods (Sambrook *et al.*, 1989).

Analysis of PHA. For quantitative and qualitative analysis of PHA, 5–7 mg lyophilized cells were subjected to methyl esterification (methanolysis) in a 1:1 (v/v) chloroform/methanol solvent mixture containing 15% (v/v) sulfuric acid. The resulting hydroxyacyl methylesters were analysed by GC as described by Timm *et al.* (1990). The retention times of authentic 3-hydroxy fatty acids were used for identification of PHA constituents.

Determination of PHA synthase activity. Determination of PHA synthase activity was done by a spectrometric assay with a 500 µl assay mixture containing 25 mM Tris/HCl, pH 7.4, 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 20 mM MgCl₂, 100 µM D(-)-3-hydroxybutyryl-CoA and approximately 30 µg protein from the soluble protein fraction of cells of *Escherichia coli*. The enzyme reactions were carried out at 30 °C and the activity estimations followed the method described by Valentin & Steinbüchel (1994).

RESULTS AND DISCUSSION

PHA accumulation in cyanobacteria

Physiological evidence for the presence of a PHA synthase in the investigated cyanobacteria was obtained by two-stage cultivation of 10 different strains of

cyanobacteria in BG11 and in nitrogen-free BG110 medium containing acetate. Among them only two fast-growing strains of *Synechococcus* sp. PCC 6715 and *Synechococcus* sp. MA19 reached exponential growth phase after 4 d. Eight strains accumulated poly(3HB) at levels ranging from 0.7 to 6.5% (w/w) of cell dry matter (CDM). For *Cyanothece* sp. strains PCC 8955 and PCC 8801 only trace amounts of poly(3HB) were determined (Table 3). In the absence of acetate, the poly(3HB) content of these strains was negligible or even undetectable (data not shown). The thermophilic cyanobacteria *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912, which grew optimally at 50 °C, accumulated by far the highest amounts (approx. 6.5 and 6.2%, w/w, of CDM, respectively) of poly(3HB). Transmission electron micrographs of thin sections revealed typical poly(3HB) granules with monolipid boundary layers in acetate-grown cells of both thermophilic cyanobacteria (data not shown). If the bactericide triclosan, known to be an inhibitor of fatty acid *de novo* synthesis (Hoang & Schweizer, 1999), was applied to the acetate-growing cultures at a concentration of 1.5 µg ml⁻¹, neither the content, the composition of the accumulated PHA (Table 3) nor the fatty acid profiles of the methanolized cells were significantly affected (data not shown). At this concentration triclosan did not affect growth of almost all strains studied, with the exception of *Cyanothece* sp. strains PCC 8303 and PCC 8801, which exhibited chlorosis of the cells after 72 h incubation. Triclosan was also tested at concentrations up to 5 µg ml⁻¹. However, at a concentration of 2.5 µg ml⁻¹ or higher it caused chlorosis and cell lysis of the cyanobacteria studied. Thus, acetate is directly metabolized to acetyl-CoA and further to poly(3HB), probably employing the *Ralstonia eutropha*

Table 3. Growth and PHA accumulation in cyanobacteria cultivated in acetate medium with and without triclosan added

Experiments were done in triplicate and the mean values are provided with standard errors ($P < 0.05$). Two-stage cultivation experiments are described in Methods. In the absence of acetate the cells accumulated negligible (trace, TR) or undetectable (ND) amounts of PHA.

Strain	PHB (% w/w, of CDM)		Effect of triclosan (1.5 µg ml ⁻¹)	
	BG110	+ Acetate	Chlorosis*	PHB (% w/w, of CDM)
<i>Chlorogloeopsis fritschii</i> PCC 6912	TR	6.2 ± 1.5	—	6.7 ± 1.0
<i>Cyanothece</i> sp. PCC 7424	TR	1.1 ± 0.1	—	0.5 ± 0.1
<i>Cyanothece</i> sp. PCC 8303	0.3 ± 0.1	2.7 ± 0.5	+	3.3 ± 0.2
<i>Cyanothece</i> sp. PCC 8801	ND	TR	+	TR
<i>Cyanothece</i> sp. PCC 8955	ND	TR	—	TR
<i>Gloeocapsa</i> sp. PCC 7428	< 0.3	1.5 ± 0.1	—	1.2 ± 0.2
<i>Gloeotheca</i> sp. PCC 6501	TR	1.4 ± 1.0	—	1.0 ± 0.1
<i>Stanieria</i> sp. PCC 7437	TR	0.9 ± 0.5	—	0.8 ± 0.1
<i>Synechococcus</i> sp. MA19	0.5 ± 0.2	6.5 ± 2.5	—	6.8 ± 0.5
<i>Synechococcus</i> sp. PCC 6715	ND	0.7 ± 0.5	—	0.5 ± 0.2

* Triclosan at a concentration of 1.5 µg ml⁻¹ did (+) or did not (—) affect growth of the cyanobacteria studied.

Table 4. Four approaches for identification of PHA synthases in cyanobacteria applied in this study

+, Detectable amounts of poly(3HB) or positive reaction; —, trace amount of poly(3HB) or negative reaction; ND, not determined. The experiments were carried out in triplicate and followed the protocols described in Methods.

Strain	Poly(3HB) accumulation	Southern blot (<i>phaC</i> probe)	Western blot (anti-PhaE _{Syn})	PCR product (bp)
<i>Anabaena cylindrica</i> SAG 1403-2*	+	+	—	600
<i>Chlorogloeopsis fritschii</i> PCC 6912	+	+	+	555
<i>Cyanothece</i> sp. PCC 7424	+	ND	+	—
<i>Cyanothece</i> sp. PCC 8303	+	+	+	550
<i>Cyanothece</i> sp. PCC 8801	—	—	+	ND
<i>Cyanothece</i> sp. PCC 8955	—	ND	—	—
<i>Gloeocapsa</i> sp. PCC 7428	+	ND	—	—
<i>Gloeotheca</i> sp. PCC 6501	+	—	—	ND
<i>Stanieria</i> sp. PCC 7437	+	ND	—	—
<i>Synechocystis</i> sp. PCC 6803*	+	+	+	560
<i>Synechococcus</i> sp. MA19	+	—	+	565
<i>Synechococcus</i> sp. PCC 6715	+	—	+	600

* PHA accumulation in *Anabaena cylindrica* SAG 1403-2 and *Synechocystis* sp. PCC 6803 was reported by Vincenzini & De Philippis (1999) and Hein *et al.* (1998).

biosynthetic pathway using β -ketothiolase and acetoacetyl-CoA reductase. Evidence for this was recently obtained by a different approach (Taroncher-Oldenburg *et al.*, 2000).

Southern hybridization with the *phaC*_{Syn} DNA probe

In further experiments we screened the genomes of the investigated cyanobacteria for the presence of PHA synthase structural genes and for the specific type of PHA synthase synthesized using various methods. A first approach to investigate the distribution of type-III PHA synthases in PHA-accumulating cyanobacteria was Southern hybridization. We focused on the type-III PHA synthase because *Synechocystis* sp. PCC 6803, which is the only cyanobacterium in which PHA synthase genes have already been identified, possesses this type of enzyme (Hein *et al.*, 1998). Cyanobacterial total genomic DNA of six strains was digested separately using *Pst*I, *Eco*RV or *Bam*HI and the DIG-labelled PCR product containing the *Synechocystis* sp. PCC 6803 PHA synthase structural gene (*phaC*_{Syn}) was employed as DNA probe. In addition to the six strains studied above, the poly(3HB)-accumulating cyanobacteria *Anabaena cylindrica* SAG 1403-2 and *Synechocystis* sp. PCC 6803 were also investigated. The results are summarized in Table 4. In addition to the positive control (*Synechocystis* sp. PCC 6803) only three genomes revealed a positive signal: *Anabaena cylindrica* SAG 1403-2, *Chlorogloeopsis fritschii* PCC 6912 and *Cyanothece* sp. PCC 8303, whereas the genomic DNA fragments of the other four strains gave no signals (Table 4). Since three of the latter were able to accumulate poly(3HB) and must therefore possess PHA synthase structural genes, the absence of hybridization might be

caused either by differences in the codon usage in the *phaC* genes of these strains as compared to that of *Synechocystis* sp. PCC 6803 or by the presence of a different type, or at least a less similar, PHA synthase.

Screening for PhaE-containing strains using His₆-tagged PhaE_{Syn} antibodies

One further approach was the application of Western immunoblotting using crude extracts of the various cyanobacteria and antibodies raised against various PHA synthases. Antibodies raised against the type-I and type-III PHA synthases from *R. eutropha* (PhaC_{Re}) and *Allochromatium vinosum* (PhaC_{Av}), respectively (Liebergesell *et al.*, 1994), gave no cross-reaction with the samples investigated in this study putatively containing cyanobacterial PHA synthases (data not shown and Hein *et al.*, 1998).

We therefore purified the PHA synthase PhaE from *Synechocystis* sp. PCC 6803 (PhaE_{Syn}). A His₆-tagged *phaE*_{Syn} (1.1 kbp) construct was made and expressed in *Escherichia coli* TOP10 under the control of *P*_L and *P*_R of the λ promoter of plasmid pMa/c5-914, the construction of which is shown in Fig. 1. The fusion protein was purified by affinity chromatography on an Ni-NTA column followed by SDS-PAGE. PhaE_{Syn}, which was eluted from the SDS-polyacrylamide gel, was verified by N-terminal amino acid sequence analysis and was submitted for antibody production. The polyclonal antibodies raised against PhaE_{Syn} were purified from rabbit serum as described in Methods.

Western blotting using the anti-PhaE_{Syn} antibodies was performed with protein crude extracts from 12 strains of cyanobacteria, including *Synechocystis* sp. PCC 6803.

As reference samples a crude extract of recombinant *Escherichia coli* expressing *PhaE_{syn}* and proteins solubilized from poly(3-hydroxybutyrate) (PHB) granules of *Synechocystis* sp. PCC 6803 (Hein *et al.*, 1998) were applied. Specific cross-reactions occurred with a 37 kDa protein in the crude protein fraction of *Synechocystis* sp. PCC 6803, in the PHB granule protein extract and in the recombinant *Escherichia coli* extract. In addition, crude extracts from *Synechococcus* sp. MA19, *Chlorogloeopsis fritschii* PCC 6912 and *Cyanotheca* PCC 8303 gave weak signals, each corresponding to a 40 kDa protein band. Moreover, two signals occurred at 36 and 40 kDa in *Cyanotheca* sp. strains PCC 7424 and PCC 8801, and also in *Synechococcus* sp. PCC 6715 (Table 4). It remains to be analysed whether these three latter cyanobacteria possess two *PhaE* subunits of different molecular masses, or whether the 36 kDa protein is a proteolysis product of the 40 kDa protein. Crude extracts from the following cyanobacteria did not reveal any protein cross-reaction with the anti-*PhaE_{syn}* antibodies: *Anabaena cylindrica* SAG 1403-2, *Cyanotheca* sp. PCC 8955, *Gloeotheca* sp. PCC 6501, *Gloeocapsa* sp. PCC 7428 and *Stanieria* sp. PCC 7437 (Table 4). It is remarkable that *Anabaena cylindrica* SAG 1403-2 obviously contains *phaC* according to hybridization with the *phaC*-specific probe, but seems to lack *PhaE* according to the result of the Western blotting experiment. Since this strain is similar to *Anabaena cylindrica* 10C (Lama *et al.*, 1996), as indicated by the accumulation of poly(3HB-co-3HV) (Vincenzini & De Philippis, 1999), *PhaE* or a homologous protein might be more diverse among the strains studied.

In conclusion, these immunological studies provide further evidence for the widespread distribution of type-III PHA synthases in the cyanobacterial strains investigated in this study. As mentioned above, the antibodies against *PhaC_{Re}* and *PhaC_{Av}* did not reveal cross-reaction with *PhaC_{syn}*. Therefore, it is clearly shown that antibodies raised against the more specific and typical components are suitable to screen for the distribution of type-III PHA synthases among cyanobacteria and probably also in other bacteria.

Screening cyanobacterial genomes for *phaC* by using PCR

A third approach used to investigate the occurrence and distribution of type-III PHA synthases in cyanobacteria employed PCR using genomic DNA of the various cyanobacteria as template and oligonucleotides specific for the *phaC* gene as primers. For this, the nucleotide sequences of the oligonucleotides were designed according to a highly conserved region of the *PhaC* superfamily. PCR products were obtained for *Chlorogloeopsis fritschii* PCC 6912 (555 bp), *Synechococcus* sp. MA19 (565 bp), *Cyanotheca* sp. PCC 8303 (550 bp), *Anabaena cylindrica* SAG 1403-2 (approx. 600 bp) and *Synechococcus* sp. PCC 6715 (approx. 600 bp). The sizes of the first three PCR products were verified by DNA sequencing, whereas the sizes of the last two

products were determined by 1.2% (w/v) agarose gel electrophoresis only. A fragment of the expected size of 560 bp was obtained for *Synechocystis* sp. PCC 6803, which again served in this study as a positive control. The minor variations in the sizes of the PCR products were not only caused by the individual nucleotide sequences of templates, but also by the positions where the degenerate primers hybridized.

The amino acid sequences deduced from the nucleotide sequences of the PCR products of *Chlorogloeopsis fritschii* PCC 6912, *Cyanotheca* sp. PCC 8303 and *Synechococcus* sp. MA19 exhibited 76, 83 and 75% identity, respectively, with the corresponding regions of *PhaC_{syn}*. Alignment of these sequences, using CLUSTAL W (1.81) software of the European Bioinformatics Institute (<http://www2.ebi.ac.uk>), showed striking homologies not only to amino acid sequences of the *PhaC* proteins of other type-III PHA synthases, but also to those of type-I and type-II PHA synthases, such as *PhaC* of *R. eutropha* and *PhaC1* of *P. aeruginosa*, respectively. These regions are considered as the covalent substrate binding regions, containing conserved cysteine residues corresponding to positions Cys-130 and Cys-149 in *PhaC* from *Allochromatium vinosum*, as shown recently by Jia *et al.* (2000). It is remarkable that these regions also contain a highly conserved Cys-157 residue and a stretch of amino acids typical for cyanobacteria that does not occur in the type-III *PhaC* proteins of anoxygenic photosynthetic bacteria. We have therefore termed this the cyanobacterial box (see Fig. 2). This region is different from the corresponding sequences of all other type-III PHA synthases and also from type-I and type-II PHA synthases.

Cloning of the *pha* loci of the thermophilic cyanobacteria *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912

The two thermophilic cyanobacteria, represented by the unicellular strain *Synechococcus* sp. MA19 and the heterocystous branching filamentous strain *Chlorogloeopsis fritschii* PCC 6912, were the most active PHA-accumulating strains (Table 3) and must obviously possess thermotolerant PHA synthases, which have been so far unavailable and which are of interest for various biotechnological applications. Therefore, all further studies focused on these PHA synthases. We applied inverse PCR to clone the entire PHA synthase structural genes from *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912. Genomic DNA isolated from these two cyanobacteria was digested with restriction enzymes *EcoRV*, *BamHI* and *PstI*, which do not cleave in *slr1829* (*phaE*) and *slr1830* (*phaC*) of *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996; Hein *et al.*, 1998). It was therefore unlikely that these restriction enzymes would have recognition sites close to the PCR products obtained above. The DNA fragments were subsequently religated using T4 DNA ligase and the recycled DNA molecules were then digested by *SspI*, for which there is a unique site in the previously sequenced

Cys-149 (*A. vinosum*)
↓

<i>R. eutropha</i>	LVSWRNPDSMAGSTWDDYIEHAARAIVEARDISGQKINVLGFCVGGTIVSTALAVLA	333
<i>P. aer</i> (PhaC1)	IVSWRNPTKSQREWLTTYIEALKE-AIEVVLSITGSKDNLNLCACSGGITATTATIGHYV	311
<i>A. vinosum</i>	LIDWGYPDQGDRALTLDYINGYIDRCVDYLREAHGVKVNNLGLICQGGAFSLMYASLHP	163
<i>T. violacea</i>	LIDWGYPDQADSALTLDYINGYIDSCVDYLCETHETQVNILGLICQGGAFSLMYASLHP	162
<i>T. pfennigii</i>	LIDWGYPDQADRALTLDYINGYIDRCVDYLRETHGVQV-LLGICQGGAFSLCYTAHLS	163
<i>E. shaposhk.</i>	LIDWGYPDRADRFLTMDDYLNGLYDRCVDEICRRHNLDKINILGLICQGGTFSLCYSAMHP	162
<i>Cyanotheca</i>	LIDWGYPGRGDRWLTLDDYINGYLNNCVDFIRASHQLDKVNNLGLICQGGTFSLCYSSLYP	169
<i>Synech.</i> MA19	LIDWGYPTRADRWLTLDYINGYINNVCDFIRKHQDLKINILLGCQGGTFSLCYSAIYP	065
<i>C. fritschii</i>	LIDWGYPGRGDRWLTLDDYINGYLNNCVDFIRTSHQLDKVNNLGLICQGGTFSLCYSSLYP	167
<i>Syn.</i> PCC 6803	LIDWGYPSRGRDWLTLEDYLSGYLNNCVDIIQRSSQQKKTLLGLWCQGGTFSLCYASLFP	178
Consensus	:::*::*: :::::::::::::::::::: : ::::::::::::::*:*:**::*: :::	

<i>R. eutropha</i>	APAASVTLLTTLLDFADTGILDVFDEGHVQLREATLGGGAGAPCALLRGLELANTFNFL	393
<i>P. aer</i> (PhaC1)	AKV-NAFTQLVSVLDFELNTQVALFADEKTLAAKKRSYQSGVLE-GKKD-MAKFVAVMR	368
<i>A. vinosum</i>	DKVNNLVMTVPVDFKTPGNLLSAW-----VQNVDIDLAVDTMGNIPEGELLNWTFLSLK	216
<i>T. violacea</i>	DKVKNLVMTVPVDFKTPGNLLSAW-----VQNVDIDLAVDTMGNIPEGELLNWTFLSLK	217
<i>T. pfennigii</i>	EKVKNLVMTVPVDFQTPGNLLSAW-----VQNVDVDLAVDTMGNIPEGELLNWTFLSLK	217
<i>E. shaposhk.</i>	EKVNLIITMVTVPFQTPDNLSHW-----VKHVIDLTPLVDTMGNVPEGLLNWTFLNLK	216
<i>Cyanotheca</i>	DKVNNLVMMVAPVDFHQPETLLNLRGGCTLGAEFIDVLMVDALGNIPGDFLNLEFLMLK	129
<i>Synech.</i> MA19	EKVKNLVMTVPVDFQISDSLILNRGGCTLGAEFIDVLMVDCLGNIPGDFLNFEFLMLK	225
<i>C. fritschii</i>	DKVNNLVMMVAPVDFHQPETLLNLRGGCTLGAEFIDVLMVDALGNIPGDFLNLEFLMLK	227
<i>Syn.</i> PCC 6803	DKVKNLVMMVAPVDFEQGTLLNLRGGCTLGAEFIDVLMVDALGNIPGDFLNLEFLMLK	238
Consensus	::::::::::: . :: ***** :::::::::::::: :::::::::::::: :::	

↓
Cyanobacterial box

Fig. 2. Similarities and differences of deduced amino acid sequences of the substrate covalent binding site of PHA synthases. The substrate-binding site, corresponding to Cys-149 of *Allochrochromatium vinosum*, of various PhaC proteins of all three types of PHA synthases were aligned by using CLUSTAL W software. Here, the partial primary structure of *Synechococcus* sp. MA19, *Chlorogloeopsis fritschii* PCC 6912, *Cyanothece* sp. PCC 8303, *Synechocystis* sp. PCC 6803, *Ectothiorhodospira shaposhnikovii* and of three purple sulfur bacteria, *Allochrochromatium vinosum*, *Thiocystis violacea* and *Thiococcus pfennigii*, were analysed. As representatives of type-I PHA and type-II PHA synthases the corresponding regions of PhaC from *R. eutropha* and PhaC1 from *Pseudomonas aeruginosa* were co-aligned. In the highly conserved region (*) of the PhaC subunits of cyanobacteria the 'cyanobacterial box' is indicated. Absolute conservation, high conservation and low conservation are indicated by '***', '**' and '*', respectively.

Fig. 3. Flow diagram describing the procedure to clone *phaE* and *phaC* from the two thermophilic cyanobacteria. Primer sequences are shown in Table 2.

PCR products. These template DNA molecules were used in combination with the primers given in Methods (see Table 2). Inverse PCR was successful only for the *Pst*I-digested DNA of strains MA19 and PCC 6912 and gave products of 2250 and 2430 bp, respectively. The inverse PCR products were ligated to *Eco*RV-restricted pSK⁺ DNA, transformed into *Escherichia coli* XL-1 Blue and were subsequently sequenced. The PCR procedures employed are shown in Fig. 3. The DNA sequences obtained were analysed by the computer programs HUSAR and BLAST as described in Methods.

Two ORFs, corresponding to *slr1830* (*phaC*_{Syn}) and *slr1829* (*phaE*_{Syn}), were identified in the inverse PCR products obtained with *Chlorogloeopsis fritschii* PCC 6912 and *Synechococcus* sp. MA19 template DNA. Both genes were separated by intragenic regions of 165 and 93 bp in the genomes of strains PCC 6912 and MA19, respectively. Unfortunately, both 5' regions of the *phaE* genes of strains MA19 (*phaE*_{MA19}) and PCC 6912 (*phaE*_{Cf}) possessed a restriction site for *Pst*I. Therefore, approximately 100 aa of the PhaE protein were missing in both strains. The amino acids deduced from the

Fig. 4. Alignment of the C-terminal amino acid sequences encoded by various *phaE* genes. The sequences of *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912, which were determined in this study, were aligned with those of *Synechocystis* sp. PCC 6803, *Allochrochmatium vinosum*, *Thiocystis violacea*, *Thiococcus pfennigii* and *Ectothiorhodospira shaposhnikovii*. The PhaE box shows a region of absolutely identical amino acids occurring among PhaE subunits. The stretches Gln-Val-Ala-Ala-Leu-Ala-Gly and Ala-Pro-Ala-Pro-Ala-Thr occurring in the PhaE proteins of anoxygenic sulfur bacteria, which might be related to PHA granule binding sites (Liebergesell *et al.*, 2000), are boxed for comparison with the corresponding regions in the PhaE proteins of cyanobacteria. Absolute conservation, high conservation and low conservation are indicated by '*', '.' and ':', respectively. n_1 and n_j are the numbers of amino acids in C-terminal regions of PhaE_{MA19} and PhaE_{Cf}, respectively.

Fig. 5. Alignment of the C-terminal amino acid sequences deduced from *phaC* from seven strains of cyanobacteria and photo-synthetic γ -Proteobacteria.

previously sequenced DNA fragments of *phaE*_{Cf} and *phaE*_{MA19} exhibited higher similarities to the PhaE proteins of anoxygenic photosynthetic bacteria than did PhaE_{Syn} to the PhaE proteins of other type-III PHA synthases. However, their C-terminal regions showed no homology to the Gln-Val-Ala-Ala-Leu-Ala-Gly stretch, which is typical of the C-terminal regions of PhaE proteins of the photosynthetic γ -Proteobacteria (Fig. 4). This stretch has been suggested to act as the binding site of the PHA synthase complex to the PHA granule surface of these bacteria (Liebergesell *et al.*, 2000). It is remarkable that the C-terminal region of PhaE_{Cf} exhibited a unique Ala-Pro-Ala-Pro-Ala-Thr stretch, which is identical to that of *Thiocystis violacea* (Fig. 4) and is related to other binding sites of phasin proteins (Liebergesell *et al.*, 2000).

Comparison of cyanobacterial PHA synthases with PHA synthases from other bacteria

PCR products from the *phaC* upstream and downstream regions of *Synechococcus* sp. MA19 (*phaC*_{MA19}) and *Chlorogloeopsis fritschii* PCC 6912 (*phaC*_{Cf}) were obtained, ligated into pSK⁻, transformed into *Escherichia coli* XL-1 Blue and sequenced. Comparison of the amino acid sequences deduced from *phaC*_{MA19} (364 aa) and *phaC*_{Cf} (366 aa) with the primary structures of proteins in the NCBI database revealed the following similarities to other PHA synthases: PhaC_{MA19} exhibited identities of 74, 67, 58, 55, 54 and 52% to the PhaC

proteins of *Chlorogloeopsis fritschii* PCC 6912, *Synechocystis* sp. PCC 6803, *Ectothiorhodospira shaposhnikovii*, *Thiocystis pfennigii*, *Allochrochmatium vinosum* and *Thiocystis violacea*, respectively; PhaC_{Cf} exhibited identities of 71, 53, 53, 53 and 52% to the PhaC proteins of the same five strains, respectively.

The high level of similarity among these sequences confirms the high level of conservation of these PhaC regions, from which the universal *phaC* screening primers, Haphapcr1 and Haphapcr2, were deduced and degenerated. However, the amino acid sequences deduced from PhaC_{MA19} and PhaC_{Cf} also exhibited highly conserved regions adjacent to the positions corresponding to Cys-149, Asp-302, His-303 and His-331 in the sequence of *Allochrochmatium vinosum*. These residues are important for activation of the 3-hydroxyalkyl moiety of 3-hydroxybutyryl-CoA (Asp-302, His-303) and nucleophilic attack (His-331) (Fig. 5), as well as for covalent catalysis carried out at Cys-149 (Jia *et al.*, 2000; see also Fig. 2). In addition to the results shown above, about 180 aa could be deduced from the *phaC*-specific PCR product obtained from *Cyanothece* sp. PCC 8303 (PhaC_{C.sp}). This partial gene sequence was also aligned with all PhaC proteins of type-III PHA synthases using the CLUSTAL W program. The amino acid sequences at the covalent catalysis site of PhaC of both thermophilic cyanobacteria and the mesophilic cyanobacterium *Cyanothece* sp. PCC 8303 also reveal striking similarities to the active centres of lipases, which exhibit a mechanism of catalysis similar to that of PHA

Fig. 6. Phylogenetic tree of PhaC subunits of type-III PHA synthases. Protein distance and bootstrap proportions were calculated from the alignment of sequences (355 aa) from the N-terminal regions of eight PhaC proteins using CLUSTAL X software and the PROTDIST and PROTPARS programs of the PHYLIP package. The sequences identified in this study are printed in bold type. The amino acid sequence of PhaC (355 aa) from *Bacillus megaterium* (McCool & Cannon, 1999) was used as outgroup. Bootstrap support for branches present in more than 50% of 1000 resamplings is indicated. A scale bar of 0.1 nt substitutions per site is shown at the bottom.

synthases (Liebergesell & Steinbüchel, 1993; Jia *et al.*, 2000).

Distance matrices were calculated using the PHYLIP program package (Felsenstein, 1989). On the basis of PROTDIST and PROTPARS analyses a phylogenetic tree was generated from each of the three PhaC sequences of cyanobacteria (*Synechocystis* sp. PCC 6803, *Synechococcus* sp. MA19, *Chlorogloeopsis fritschii* PCC 6912) and anoxygenic photosynthetic γ -Proteobacteria (*Thiococcus pfennigii*, *Allochromatium vinosum*, *Thiocystis violacea* and *Ectothiorhodospira shaposhnikovii*). *Bacillus megaterium* PhaC was used as outgroup. In the cyanobacterial subgroup, PhaC of *Synechocystis* sp. PCC 6803 separated from the PhaC proteins of the two thermophilic strains *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912. In the anoxygenic photosynthetic bacteria subgroup PhaC of *Ectothiorhodospira shaposhnikovii* was separated from the PhaC proteins of *Thiococcus pfennigii*, *Thiocystis violacea* and *Allochromatium vinosum* (Fig. 6).

Co-expression of *phaC*_{MA19} or *phaC*_{Cf} with *phaA* and *phaB* of *Synechocystis* sp. PCC 6803

It has been reported previously that functionally active poly(3HB) biosynthesis pathways relying on type-III PHA synthases are heterologously expressed in *Escherichia coli* only if both subunits PhaE and PhaC from one strain (Hein *et al.*, 1998; Taroncher-Oldenburg *et al.*, 2000) or from two different strains (Liebergesell *et al.*, 2000) are co-expressed together with *phaA* and *phaB*, encoding β -ketothiolase and acetoacetyl-CoA reductase, respectively, from *R. eutropha* or from *Synechocystis* sp. PCC 6803. The corresponding recombinant strains of *Escherichia coli* accumulated poly(3HB) amounting to 13% (w/v) of CDM when the cells were cultivated in LB medium containing 1% (w/v) glucose. In the case of the PHA synthase of *Allochromatium vinosum*, the enzyme was only active if both subunits PhaC and PhaE were co-expressed with PhaA and PhaB (Liebergesell *et al.*, 1994; Müh *et al.*, 1999). Since the *phaE-phaC* intragenic regions in the genomes of *Synechococcus* sp. MA19 and *Chlorogloeopsis fritschii* PCC 6912 are longer than in other bacteria possessing type-III PHA synthases, *phaC*_{MA19} and *phaC*_{Cf} were co-expressed together with *phaA-B*_{Syn} in *Escherichia coli* XL-1 Blue to investigate whether PhaC from both strains conferred PHA synthase activity *in vivo* as well as *in vitro*. A recombinant strain of *Escherichia coli* harbouring the hybrid plasmid pSKABC_{MA19} (Fig. 7), in which *phaAB*_{Syn} was located downstream of *phaC*_{MA19} and in co-linear orientation to the *lacZp/o*, exhibited an *in vitro* activity of 0.8 U (mg protein)⁻¹, whereas cells harbouring plasmid pSKAB_{Syn} (Fig. 7) exhibited a residual activity of only 0.45 U (mg protein)⁻¹. Using a similar construction for PhaC_{Cf}, a specific PHA synthase activity of 1.1 U (mg protein)⁻¹ was measured with pSKABC_{Cf} (Fig. 7). As a positive control the PHA synthase in pSKABEC_{Syn} exhibited about 10 U (mg protein)⁻¹ (Table 5). The recombinant cells harbouring pSKABC_{MA19} or pSKABC_{Cf} accumulated poly(3HB) amounting to 2 and 1.5% (w/w) of CDM, respectively, if grown in LB containing 0.6% (w/v) glucose, 0.2 mM IPTG and 50 μ M thiamine. The methanolized substances from the cells harbouring the control plasmid pSKAB_{Syn} and grown under the same conditions also contained a 3HB peak amounting to 0.8% (w/w) of CDM and most probably representing monomers of 3HB. In the absence of IPTG, the poly(3HB) content of the cells was only 0.2% (w/w) of CDM (Table 5). These experiments demonstrated that *phaC* of the investigated cyanobacteria alone is able to confer low but significant residual PHA synthase activity and poly(3HB) synthesis to *Escherichia coli* in the absence of *phaE*. However, this only occurred if β -ketothiolase (PhaA) and acetoacetyl CoA reductase (PhaB) were also expressed. It should be noted that it has been shown that the purified PhaC protein of *Allochromatium vinosum* exhibited approximately 0.6% residual PHA synthase activity of the intact PHA synthase consisting of PhaE plus PhaC (Müh *et al.*, 1999). This is significantly less than the PHA synthase

Fig. 7. Construction of hybrid plasmid pSKABC_{MA19} for investigating the *in vitro* activity of PhaC_{MA19} by co-expression with PhaA_{Syn} and PhaB_{Syn} of *Synechocystis* sp. PCC 6803. A similar construct was made to obtain the corresponding plasmids pSKABC_{Cf} and pSKABEC_{Syn} to study the activity of the PHA synthase of *Chlorogloeopsis fritschii* PCC 6912 and as a positive control, respectively.

Table 5. PHA accumulation and *in vitro* PHA synthase activity of recombinant strains of *Escherichia coli*

ND, Not detectable; NA, not analysed.

Strain (plasmid)	Relevant markers	IPTG (0.2 mM)	Poly(3HB) (%, w/w, of CDM)	Specific activity [U (mg protein) ⁻¹]*
<i>Escherichia coli</i> XL-1 Blue	See Table 1	—	ND	ND
pSK ⁻	See Table 1	+	ND	< 0.10
pSKC _{MA}	<i>phaC</i> _{MA19} ⁺	+	ND	< 0.10
pSKC _{Cf}	<i>phaC</i> _{Cf} ⁺	+	ND	< 0.10
pSKAB _{Syn}	<i>phaA</i> _{Syn} ⁺ <i>phaB</i> _{Syn} ⁺	+	0.8 ± 0.4	0.45 ± 0.15
pSKABC _{MA}	<i>phaA</i> _{Syn} ⁺ <i>phaB</i> _{Syn} ⁺ <i>phaC</i> _{MA19} ⁺	+	2.0 ± 0.1	0.80 ± 0.05
pSKABC _{MA}	<i>phaA</i> _{Syn} ⁺ <i>phaB</i> _{Syn} ⁺ <i>phaC</i> _{MA19} ⁺	—	0.2 ± 0.1	NA
pSKABC _{Cf}	<i>phaA</i> _{Syn} ⁺ <i>phaB</i> _{Syn} ⁺ <i>phaC</i> _{Cf} ⁺	+	1.5 ± 0.2	1.1 ± 0.10
pSKABEC _{Syn}	<i>phaA</i> _{Syn} ⁺ <i>phaB</i> _{Syn} ⁺ <i>phaE</i> _{Syn} ⁺ and <i>phaC</i> _{Syn} ⁺	+	43.0 ± 1.5	10.0 ± 0.50

* One unit (U) is defined as 1 μmol substrate [D-(—)-3-hydroxybutyryl-CoA] min⁻¹.

activity conferred to *Escherichia coli* by the cyanobacterial PhaC proteins investigated in this study.

Conclusions

The cyanobacterial strains investigated in this study accumulated PHA only in the presence of acetate. Under these mixotrophic growth conditions 3HB was the only

constituent of the accumulated polyester. Multiple evidence was obtained that biosynthesis of poly(3HB) in the investigated cyanobacteria is due to a type-III PHA synthase. Evidence for this was obtained by Southern blot analysis using type-III PhaC-specific DNA probes, Western blot analysis using specific anti-PhaE_{Syn} antibodies, PCR techniques using *phaC*-specific oligomers as primers and by cloning and DNA sequence analysis of

the PHA synthase structural genes from three different cyanobacteria. Together with the data obtained previously for the PHA synthase of *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996; Hein *et al.*, 1998) and considering the phylogenetic positions of the cyanobacteria, investigated with respect to the molecular and biochemical basis of poly(3HB) biosynthesis, it can be concluded that type-III PHA synthase is probably the characteristic PHA synthase of cyanobacteria in general.

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