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

T. Hai, S. Hein and A. Steinbüchel

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 *Gloeothece* 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, thermostolerant enzymes, PHB, bioplastic, *Anabaena cylindrica*, *Gloeoeoca* sp.

INTRODUCTION

Cyanobacteria are oxygenic, photoautotrophic prokaryotes which possess two photosystems (PSI and PSII) 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), Gloeothecae 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 & Valentín, 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 microorganisms (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 Allochromatium vinosum (Liebergesell & Steinbüchel, 1992; Liebergesell et al., 1994), Thiocystis violacea (Liebergesell & Steinbüchel, 1993), Thiococcus (formerly Thiocapsa) pfnemigii (Liebergesell et al., 2000) and Ectothiorhodospira shaposhnikovi (Genbank accession no. AAG30259 for phaC and AAG30260 for phaE). These enzymes belong to the type-III PHA synthases characteristic of β-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; Mühl 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 (Tszukuba, Ibaraki, Japan). Anabaena cylindrica SAG 1403-2 was obtained from the Sammlung von Algenkulturen Göttingen (Göttingen, Germany), whereas Chlorogloea 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 µE s 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 µE m⁻² 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 µE m⁻² s⁻¹) for a further 7 d with shaking. The medium volume was adjusted daily with sterile double-distilled water during cultivation.

Triclosan (1–5 µg 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 µg triclosan 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 CaCl₂ 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
Occurrence of type-III PHA synthase in cyanobacteria

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype characteristics*</th>
<th>Reference or source†</th>
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<tr>
<td><strong>Cyanobacteria</strong></td>
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<td><em>Anabaena</em> <em>cylindrica</em> SAG 1403-2</td>
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<td>SAG</td>
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<td><em>Chlorogloeopsis fritschii</em> PCC 6912</td>
<td>Optimal growth at 50°C</td>
<td>PCC</td>
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<td>PCC</td>
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<td><em>Cyanothece</em> sp. strain PCC 8303</td>
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<td>PCC</td>
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<td><em>Cyanothece</em> sp. strain PCC 8801</td>
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<td>PCC</td>
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<td><em>Synechocystis</em> sp. strain MA19</td>
<td>Optimal growth at 50°C</td>
<td>Miyake <em>et al.</em> (1996)</td>
</tr>
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<td><em>Synechococcus</em> sp. strain PCC 6715</td>
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<td>PCC</td>
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<td>(<em>p80 lacZD15</em>)</td>
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</tr>
<tr>
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<td>Stratagene</td>
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<tr>
<td></td>
<td>[F′ proAB lacPZDM15, Tn10(Tet’)]</td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td><em>thi1 proA hsdR17 hsdM</em> <em>recA RP4-tra-function</em></td>
<td>Simon <em>et al.</em> (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
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<td><em>amp lacZp/o, T7 &amp; T3 promoters</em></td>
<td>Stratagene</td>
</tr>
<tr>
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<td>SK 6772</td>
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<td>This study</td>
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<tr>
<td>pSK::<em>phaC</em>MA19</td>
<td>1-kb PCR product of <em>phaC</em>MA19 inserted in pSK−</td>
<td>This study</td>
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<tr>
<td>pSK::<em>phaC</em>ct</td>
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<td>This study</td>
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<tr>
<td>pSKABsyn</td>
<td>3-kb PCR product of <em>phaA</em> and <em>phaB</em> (<em>phaA</em>-Bsyn) from <em>Synechocystis</em> sp. PCC 6803</td>
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<td>pSKABcct</td>
<td>3-kb PCR product of <em>phaA</em>-Bct and <em>phaC</em>ct from <em>Chlorogloeopsis fritschii</em> PCC 6912</td>
<td>This study</td>
</tr>
<tr>
<td>pSKABcMA</td>
<td>3-kb PCR product of <em>phaA</em>-BMA and <em>phaC</em>MA from <em>Synechococcus</em> sp. MA19</td>
<td>This study</td>
</tr>
<tr>
<td>pSKABECsyn</td>
<td>3-kb PCR product of <em>phaA</em>, <em>phaB</em>, <em>phaE</em> and <em>phaC</em> from <em>Synechocystis</em> sp. PCC 6803</td>
<td>This study</td>
</tr>
</tbody>
</table>

* 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-nyl 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 HisphaE<sup>syn</sup> as sense and PhaE<sup>rev</sup> 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<sup>syn</sup>*-specific PCR product obtained was purified and ligated into pMa/c5-914 (Table 1), which harbours, besides other genes, the c1857ts gene encoding the
PCR primers for the cloning of His$_{6}$-tagged phaE$_{syn}$ with inserted restriction sites for NdeI and EcoRI (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 BamHI and ApaI, and NdeI and BamHI 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5' → 3')</th>
<th>Application</th>
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<tbody>
<tr>
<td>HisphaE$_{syn}$</td>
<td>CATATGCATCACACCATCACCATCACATGGAAATCGACA</td>
<td>Overexpression</td>
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<tr>
<td>PhaEreverse</td>
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<td>Overexpression</td>
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<td>Haphapcr1</td>
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<td>Haphapcr2</td>
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<td>PhaCe69R</td>
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<td>MaphaC sense</td>
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<td>MaphaCreverse</td>
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<td>CfphaCreverse</td>
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<tr>
<td>PhaABsense</td>
<td>AGCTTTGAATTTCCATATGCCCCGC</td>
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<tr>
<td>PhaABreverse</td>
<td>TTAGGATCCGTGGGCCCCTTTTAC</td>
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</table>

![Fig. 1. Construction of the overexpression plasmid pMa/c5-914::phaE.](image)

The His$_{6}$-tagged phaE$_{syn}$ was inserted into vector pMa/c5-914 harbouring a temperature-sensitive λ repressor gene (cI857ts) 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 employing a Ni-NTA matrix chromatography column, as described in Methods. fdT indicates the transcription terminator of phage fd.

**Primer**

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<tr>
<td>PhaABreverse</td>
<td>TTAGGATCCGTGGGCCCCTTTTAC</td>
<td>Overexpression</td>
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**Table 2. Primers used in this study**

Temperature-sensitive λ repressor. The construction of the hybrid plasmid pMa/c5-914::EC1.1 is shown in Fig. 1. The His$_{6}$-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 µg ml$^{-1}$) to an OD$_{600}$ 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 passages 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 His$_{6}$-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 Sonopuls 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% (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 & Schuell) 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 *Allochromatium vinosum* and *Thioctysis violacea*, and the cyanobacterium *Synechocystis* sp. PCC 6803. The regions corresponded to amino acid positions 85–97 and 275–283, 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 2 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* XL-1 Blue 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 pphaC, respectively, were used for *Synechococcus* sp. MA19 DNA. Similarly, PhaC69R and PhaC69L (Table 2), corresponding to base positions 643–666 and 302–323 in pphaC, 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 *PstI*, cyclic products were then obtained by incubation with T4 DNA ligase and these were linearized by digestion with *SspI*. 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 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 BamHI and Apal, or NdeI and BamHI restriction sites for the genes of strains MA19 (pphaCMA19) and PCC 6912 (pphaCM19), respectively. The primers MapHaCsense and MapHaCreverse were used for cloning pphaCMA19 whereas the primers ChHisphaCs with a His6 tag and CflphaCreverse were used to clone pphaCM19 (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 *slr*1993, encoding β-ketothiolase (*phaS*), together with *slr*1994, encoding acetocacyl CoA reductase (*phaB*), from *Synechocystis* sp. PCC 6803 was amplified by using primers PhaBSsense and PhaBBreverse, which included degenerate NdeI and BamHI restriction sites, respectively (Table 2). The purified PCR product (2.45 kbp) was cloned to obtain pSKABS (see Fig. 7). After digestion with BamHI, the purified pphaAB *phab* fragment was ligated to the BamHI-treated PCR product of pphaCM19 giving the pphaABC fragment. (ii) The purified pphaABC was ligated to EcoRV-digested pSK*. The hybrid plasmid obtained, pSKABCMA19, was transformed into *Escherichia coli* XL-1 Blue. Similarly, other hybrid plasmids were constructed, such as pSKABC and pSKABECSM19 containing fragment *phaECB* which was obtained in a previous study (Hein et al., 1998). The hybrid plasmid pSKABEGCSM19 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 thermostsequenase 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 (HUAS, 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 corresponded 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 *Allochromatium 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
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 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 methyl esters 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 2,4-dinitrophenylhydrazine in the presence of 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 Valentín & 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 BG11o 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 Cyanotheca sp. strains PCC 8955 and PCC 8801 only trace amounts of poly(3HB) were determined (Table 3).

When 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. 6–7%, w/w, of CDM) 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 bacteriocide triclosan, known to be an inhibitor of fatty acid de novo synthesis (Huang & 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 Cyanotheca 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 pathway.
Occurrence of type-III PHA synthase in cyanobacteria

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Poly(3HB) accumulation</th>
<th>Southern blot (phaC probe)</th>
<th>Western blot (anti-PhaESyn)</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena cylindrica</em> SAG 1403-2*</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>600</td>
</tr>
<tr>
<td>Chlorogloeopsis fritschii PCC 6912</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>555</td>
</tr>
<tr>
<td>Cyanobacter sp. PCC 7424</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Cyanobacter sp. PCC 8303</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Cyanobacter sp. PCC 8801</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Cyanobacter sp. PCC 8955</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gloeocapsa sp. PCC 7428</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gloeobacter sp. PCC 6501</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Stanieria sp. PCC 7437</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. PCC 6803*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>560</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. MA19</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>565</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. PCC 6715</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>600</td>
</tr>
</tbody>
</table>

*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 phaCsyn 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, *EcoRV* or *BamHI* and the DIG-labelled PCR product containing the *Synechocystis* sp. PCC 6803 PHA synthase structural gene (phaCsyn) 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 *Cyanobacter* 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 PhaESyn 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<sub>R</sub>) and *Allochromatium vinosum* (PhaC<sub>Av</sub>), respectively (Liebergessell *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 (PhaESyn). A His<sub>6</sub>-tagged phaESyn (1-1 kbp) construct was made and expressed in *Escherichia coli* TOP10 under the control of P<sub>i</sub> and P<sub>β</sub> of the i 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. PhaESyn, 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 PhaESyn were purified from rabbit serum as described in Methods.

Western blotting using the anti-PhaESyn 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<sub>syn</sub> 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 *Cyanoto-
thece* PCC 8303 gave weak signals, each corresponding
to a 40 kDa protein band. Moreover, two signals
occurred at 36 and 40 kDa in *Cyanothecae* 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<sub>syn</sub>
antibodies: *Anabaena cylindrica* SAG 1403-2, *Cyanoto-
thece* PCC 8955, *Gloeocapsa* 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 investi-
gated in this study. As mentioned above, the antibodies
against PhaC<sub>Re</sub> and PhaC<sub>Av</sub> did not reveal cross-reaction
with PhaC<sub>syn</sub>. 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 proba-
bly 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 ac-
cording to a highly conserved region of the PhaC
superfamily. PCR products were obtained for *Chloro-
gloeopsis fritschii* PCC 6912 (555 bp), *Synechococcus*
sp. MA19 (565 bp), *Cyanothecae* 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, *Cyanothecae* sp. PCC 8303 and
*Synechococcus* sp. MA19 exhibited 76, 83 and 75%
identity, respectively, with the corresponding regions
of PhaC<sub>syn</sub>. 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 con-
served 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 cor-
responding 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 *Chloro-
gloeopsis 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 synthe structural
genes from *Synechococcus* sp. MA19 and *Chloro-
gloeopsis fritschii* PCC 6912. Genomic DNA isolated
from these two cyanobacteria was digested with re-
striction enzymes EcoRV, BamHI and PstI, which do not
cleave in *slr1829* (*phaE*) and *slr1830* (*phaC*) of *Synecho-
cystis* 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
Occurrence of type-III PHA synthase in cyanobacteria

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**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 Allochromatium 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 fritschi PCC 6912, Synechocystis sp. PCC 8603, Ectothiorhodospira shaposhnikovii, and of three purple sulfur bacteria, Allochromatium 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.

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

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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 PstI-digested DNA of strains MA19 and PCC 6912 and gave products of 2250 and 2430 bp, respectively. The inverse PCR products were ligated to EcoRV-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<sub>syn</sub>) and slr1829 (phaE<sub>syn</sub>), were identified in the inverse PCR products obtained with Chlorogloeopsis fritschi 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 S<sup>r</sup> regions of the phaE genes of strains MA19 (phaE<sub>MA19</sub>) and PCC 6912 (phaE<sub>Cf</sub>) possessed a restriction site for PstI. Therefore, approximately 100 aa of the PhaE protein were missing in both strains. The amino acids deduced from the
previously sequenced DNA fragments of \( \text{phaE} \) and \( \text{phaE}_{\text{MA19}} \) exhibited higher similarities to the PhaE proteins of anoxygenic photosynthetic bacteria than did PhaE\( \text{Syn} \) 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 \( \gamma \)-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 \( \text{phaE}_{\text{MA19}} \) exhibited a unique Ala-Pro-Ala-Pro-Ala-Thr C-terminal region of PhaE proteins of anoxygenic sulfur bacteria, which might be related to PHA granule binding sites (Liebergesell et al., 2000).

The high level of similarity among these sequences confirms the high level of conservation of these PhaC regions, from which the universal \( \text{phaC} \) screening primers, Haphapcr1 and Haphapcr2, were deduced and degenerated. However, the amino acid sequences deduced from PhaC\( \text{MA19} \) and PhaC\( \text{Cf} \) also exhibited highly conserved regions adjacent to the positions corresponding to Cys-149, Asp-302, His-303 and His-331 in the sequence of \( \text{Allochromatium vinosum} \). These residues are important for activation of the 3-hydroxyalkyl moiety of 3-hydroxybutyryl-CoA (Asp-302, His-303 and His-331) and 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 \( \text{phaC} \)-specific PCR product obtained from \( \text{Cyanothece} \) sp. PCC 8303 (Asp-302, His-303 and His-331 in the sequence of \( \text{Allochromatium vinosum} \)). This partial gene sequence was also aligned with all PhaC proteins of type-III PHA synthases using the clustalw program. The amino acid sequences at the covalent catalysis site of PhaC of both thermophilic cyanobacteria and the mesophilic cyanobacterium \( \text{Cyanothece} \) sp. PCC 8303 also reveal striking similarities to the active centres of lipases, which exhibit a mechanism of catalysis similar to that of PHA proteins of \( \text{Synechocystis} \) sp. PCC 6803, \( \text{Ectothiorhodospira shaposhnikovii} \), \( \text{Thiocystis violacea} \) and \( \text{Allochromatium vinosum} \) and \( \text{Thiocystis pfennigii} \). 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. \( n_i \) and \( n_j \) are the numbers of amino acids in C-terminal regions of PhaE\( \text{MA19} \) and PhaE\( \text{Cf} \), respectively.

\( \text{phaC} \)

Comparison of cyanobacterial PHA synthases with PHA synthases from other bacteria

PCR products from the \( \text{phaC} \) upstream and downstream regions of \( \text{Synechococcus} \) sp. MA19 (\( \text{phaC}_{\text{MA19}} \)) and \( \text{Chlorogloeopsis fritschi} \) PCC 6912 (\( \text{phaC}_{\text{Cf}} \)) were obtained, ligated into pSK\( \text{-} \) and sequenced. Comparison of the amino acid sequences deduced from \( \text{phaC}_{\text{MA19}} \) (364 aa) and \( \text{phaC}_{\text{Cf}} \) (366 aa) with the primary structures of proteins in the NCBI database revealed the following similarities to other PHA synthases: PhaC\( \text{MA19} \) exhibited identities of 74, 67, 58, 55, 54 and 52% to the PhaC proteins of \( \text{Synechococcus} \) sp. PCC 6803, \( \text{Ectothiorhodospira shaposhnikovii} \), \( \text{Thiocystis violacea} \), \( \text{Allochromatium vinosum} \) and \( \text{Thiocystis pfennigii} \). It is remarkable that the C-terminal region of \( \text{phaC}_{\text{Cf}} \) (366 aa) and \( \text{phaC}_{\text{Cf}} \) (366 aa) with the primary structures of proteins in the NCBI database revealed the following similarities to other PHA synthases: PhaC\( \text{MA19} \) exhibited identities of 74, 67, 58, 55, 54 and 52% to the PhaC proteins of \( \text{Synechococcus} \) sp. PCC 6803, \( \text{Ectothiorhodospira shaposhnikovii} \), \( \text{Thiocystis violacea} \), \( \text{Allochromatium vinosum} \) and \( \text{Thiocystis pfennigii} \).
synthases (Liebergessell & 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 phaCMA19 or phaC2 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 (Liebergessell et al., 2000) are co-expressed together with phaA and phaB, encoding β-ketothiolase and acetacetyl-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 (Liebergessell 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, phaCMA19 and phaC2 were co-expressed together with phaA–BSyn 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 pSKABCMA19 (Fig. 7), in which phaABSyn was located downstream of phaCMA19 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 pSKABCSyn (Fig. 7) exhibited a residual activity of only 0·45 U (mg protein)$^{-1}$. Using a similar construction for PhaC2, a specific PHA synthase activity of 1·1 U (mg protein)$^{-1}$ was measured with pSKABC2 (Fig. 7). As a positive control the PHA synthase in pSKABCSyn exhibited about 10 U (mg protein)$^{-1}$ (Table 5). The recombinant cells harbouring pSKABCMA19 or pSKABCSyn 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 μM thiamine. The methanolized substances from the cells harbouring the control plasmid pSKABCSyn 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 acetacetyl-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. 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.](image-url)
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

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

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Relevant markers</th>
<th>IPTG (0–2 mM)</th>
<th>Poly(3HB) (%) w/w of CDM</th>
<th>Specific activity [U (mg protein)−1]†</th>
</tr>
</thead>
</table>
| *Escherichia coli*  
XL-1 Blue | See Table 1 | – | ND | ND |
| pSK− | See Table 1 | + | ND | < 0–10 |
| pSKC MA | phaC MA+ | + | ND | < 0–10 |
| pSKC Cf | phaC CF+ | + | ND | < 0–10 |
| pSKAB Syn | phaA Syn+ phaB Syn | + | 0·8 ± 0·4 | 0·45 ± 0·15 |
| pSKABC MA | phaA Syn+ phaB Syn+ phaC MA+ | + | 2·0 ± 0·1 | 0·80 ± 0·05 |
| pSKABC MA | phaA Syn+ phaB Syn+ phaC MA+ | – | 0·2 ± 0·1 | NA |
| pSKABC CF | phaA Syn+ phaB Syn+ phaC CF+ | + | 1·5 ± 0·2 | 1·1 ± 0·10 |
| pSKABEC Syn | phaA Syn+ phaB Syn+ phaESyn+ and phaC Syn+ | + | 43·0 ± 1·5 | 10·0 ± 0·50 |

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

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**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 pSKABE CF to study the activity of the PHA synthase of *Chlorogloeopsis fritschii* PCC 6912 and as a positive control, respectively.
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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