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Polyhydroxyalkanoate (PHA) Accumulation in Sulfate-Reducing Bacteria and Identification of a Class III PHA Synthase (PhaEC) in *Desulfococcus multivorans*

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Seven strains of sulfate-reducing bacteria (SRB) were tested for the accumulation of polyhydroxyalkanoates (PHAs). During growth with benzoate *Desulfonema magnum* accumulated large amounts of poly(3-hydroxybutyrate) [poly(3HB)]. *Desulfosarcina variabilis* (during growth with benzoate), *Desulfobotulus sapovorans* (during growth with caproate), and *Desulfobacterium autotrophicum* (during growth with caproate) accumulated poly(3HB) that accounted for 20 to 43% of cell dry matter. *Desulfobotulus sapovorans* and *Desulfobacterium autotrophicum* also synthesized copolyesters consisting of 3-hydroxybutyrate and 3-hydroxyvalerate when valerate was used as the growth substrate. *Desulfovibrio vulgaris* and *Desulfotalea psychrophila* were the only SRB tested in which PHAs were not detected. When total DNA isolated from *Desulfococcus multivorans* and specific primers deduced from highly conserved regions of known PHA synthases (PhaC) were used, a PCR product homologous to the central region of class III PHA synthases was obtained. The complete *pha* locus of *Desulfococcus multivorans* was subsequently obtained by inverse PCR, and it contained adjacent $phaE_{Dm}$ and $phaC_{Dm}$ genes. $PhaC_{Dm}$ and $PhaE_{Dm}$ were composed of 371 and 306 amino acid residues and showed up to 49 or 23% amino acid identity to the corresponding subunits of other class III PHA synthases. Constructs of $phaC_{Dm}$ alone (pBBRMCS-2::$phaC_{Dm}$) and of $phaE_{Dm}C_{Dm}$ (pBBRMCS-2::$phaE_{Dm}C_{Dm}$) in various vectors were obtained and transferred to several strains of *Escherichia coli*, as well as to the PHA-negative mutants PHB$^-$4 and GPp104 of *Ralstonia eutropha* and *Pseudomonas putida*, respectively. In cells of the recombinant strains harboring $phaE_{Dm}C_{Dm}$ small but significant amounts (up to 1.7% of cell dry matter) of poly(3HB) and of PHA synthase activity (up to 1.5 U/mg protein) were detected. This indicated that the cloned genes encode functionally active proteins. Hybrid synthases consisting of $PhaC_{Dm}$ and PhaE of *Thiococcus pfennigii* or *Synechocystis* sp. strain PCC 6308 were also constructed and were shown to be functionally active.

Sulfate-reducing bacteria (SRB) are anaerobic bacteria that couple the oxidation of simple organic compounds (e.g., lactate) or of molecular hydrogen to the reduction of sulfate, which is the dominant electron acceptor in anaerobic zones of marine ecosystems, yielding the conspicuous end product hydrogen sulfide. SRB contribute significantly to the global cycling of carbon and sulfur, mineralizing more than 50% of the organic carbon via sulfate reduction in marine sediments (15, 16). In addition, they also occur in brackish and freshwater habitats. SRB represent different genera and species, most of which are affiliated with the δ subgroup of the *Proteobacteria*, and they display a diversity of nutritional and catabolic properties (30, 45, 47). Besides their ecological role in carbon and sulfur cycles, SRB are also economically relevant, since they are implicated in corrosion of iron, concrete, and other materials (26). Despite a strong interest in the metabolic properties of SRB, to our knowledge the capacity to produce storage compounds, such as polyhydroxyalkanoates (PHAs), has not been studied in detail in this important group of bacteria. The first indication of the presence of storage compounds in SRB was derived from microscopic examination. *Desulfonema magnum* appeared to be filled with granules that were suggested to be composed of poly(3-hydroxybutyric acid) [poly(3HB)] (46). *Desulfonema magnum* is a large (length, 9 to 13 μm) bacterium displaying gliding movement. Recent studies with genus-specific fluorescent in situ hybridization probes demonstrated the association of *Desulfonema* species with the filamentous sulfur-oxidizing bacterium *Thioploca*, indicating the ecological significance of these organisms in marine sediments and microbial mats (9). Other SRB that are abundant in marine sediments belong to the *Desulfococcus-Desulfosarcina* group or to the genus *Desulfovibrio* (22). *Desulfococcus multivorans* and *Desulfobotulus sapovorans* (formerly *Desulfovibrio sapovorans*) also appeared to contain granules of poly(3HB) (45). However, SRB have never been investigated in detail with respect to PHA metabolism, and even the previous assignment of cytoplasmic inclusions to poly(3HB) granules was not based on chemical analysis.

Bacteria synthesize a wide range of different PHAs, and approximately 150 different constituents of PHAs have been identified (39). Generally, PHA_{SCL} and PHA_{MCL}, consisting of short-chain and medium-chain hydroxyalkanoic acids, respectively, are distinguished. PHA synthases, which are the key enzymes of PHA biosynthesis, exhibit a very broad substrate spectrum and are capable of using a wide range of different hydroxyacyl coenzyme A (hydroxyacyl-CoA) thioesters as substrates for synthesis of these polyoxoesters. In addition, PHA synthases are also capable of biosynthesis of polythioesters,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant markers and characteristics	Source or reference
Escherichia coli strains		
TOP10	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	Invitrogen, San Diego, Calif.
S17-1	*relA1* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *thi1 proA hsdR17 hsdM*$^+$ *recA*, RP4-*tra* function	36
Ralstonia eutropha PHB$^-$ 4	PHA-negative mutant of wild-type strain H16	35
Pseudomonas putida GPp104	PHA-negative mutant of *P. putida* KT2440	13
Plasmids		
pBluescript SK$^-$	Amp^r, *lacPOZ'*, T7 and T3 promoter	Stratagene, San Diego, Calif.
pBBR1-MCS2	Km^r, *lacPOZ' mob*$^+$, broad host range	18
pHC79	Tc^r Km^r, cosmid	12
pSK15	pSK$^-$ harboring a 750-bp PCR product from *D. multivorans* DNA	This study
pSK33	pSK$^-$ harboring a 1.1-kbp PCR product from *Desulfococcus multivorans* DNA	This study
pSK6C	pSK$^-$ harboring a 2.4-kbp PCR product from *Desulfococcus multivorans* DNA	This study
pSK$phaE_{Dm}C_{Dm}$	pSK$^-$ harboring a 2.4-kbp insert with $phaE_{Dm}C_{Dm}$	This study
pSK$phaC_{Dm}$	pSK$^-$ harboring a 1.2-kbp insert with $phaC_{Dm}$	This study
pSelect::B28RV	pSelect harboring a 1.2-kbp insert with $phaE_{Tp}$	21
pBBRMCS-2 $phaC_{Dm}$	pBBRMCS-2 harboring $phaC_{Dm}$	This study
pBBRMCS-2 $phaE_{Dm}C_{Dm}$	pBBRMCS-2 harboring 2.4-kbp $phaE_{Dm}C_{Dm}$	This study
pSK$^-$::$phaE_{Syn}$	pSK$^-$ harboring *phaE* from *Synechocystis* sp. strain PCC6803	11
pSK::$phaE_{Tp}$	pSK$^-$ harboring $phaE_{Tp}$ from *Thiococcus pfennigii* subcloned from $phaEC_{Tp}$	21
pSK$^-$::AB_{Syn}	pSK$^-$ harboring *phaA* and *phaB* ($phaAB_{Syn}$) from *Synechocystis* sp. strain PCC6803	10
pBBR1MCS-2::AB_{Syn}	pBBR1MCS-2 harboring $phaAB_{Syn}$	This study
pSK$E_{Tp}C_{Dm}$	pSK$^-$ harboring $phaE_{Tp}C_{dm}$	This study
pSK$E_{Syn}C_{Dm}$	pSK$^-$ harboring $phaE_{Syn}C_{Dm}$	This study

which are in contrast to polyoxoesters composed of mercaptoalkanoic acids (23, 24). At present, all known PHA synthases can be assigned to one of four different classes according to their substrate ranges and their subunit compositions (32). PHAs have attracted much interest because they are biodegradable thermoplastics or elastomers which could be used for many applications in industry, medicine, pharmacy, and other areas (see references 1 and 6 for reviews). Therefore, it should be interesting to understand PHA metabolism and to identify the PHA biosynthesis genes in SRB. SRB are certainly not suitable for establishing processes for PHA production due to their slow growth and low cell yields; however, if their genomes contain PHA synthases that exhibit interesting properties, the genes could be expressed in other hosts, yielding interesting biotechnological processes for more efficient PHA production or novel PHAs (40, 41). In addition, knowledge about the metabolism of the abundant carbon and energy storage compound PHA in SRB might eventually provide insights into survival strategies when substrate availability in natural habitats is changing.

In the present study, we determined the chemical compositions of the assumed storage compounds in the three abovementioned SRB. In addition, we included *Desulfosarcina variabilis* and three SRB that were recently selected for genome sequencing, namely, *Desulfobacterium autotrophicum* (www.regx.de), *Desulfotalea psychrophila* (www.regx.de), and *Desulfovibrio vulgaris* (www.tigr.org). We also identified and characterized the PHA synthase structural genes of *Desulfococcus multivorans* and expressed the cloned genes heterologously in other bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The SRB *Desulfococcus multivorans* DSM 2059, *Desulfobotulus sapovorans* (formerly *Desulfovibrio sapovorans*) DSM 2055, *Desulfosarcina variabilis* DSM 2060, *Desulfonema magnum* DSM 2077, *Desulfobacterium autotrophicum* HRM2 (= DSM 3382), *Desulfovibrio vulgaris* Hildenborough (= DSM 644), and *Desulfotalea psychrophila* LSv54 (= DSM 12343) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). All other bacteria and plasmids used in this study are listed in Table 1.

Media and cultivation conditions. SRB were grown in bicarbonate-buffered, sulfide-reduced mineral media as previously described (45). Cultivation of *Desulfosarcina variabilis*, *Desulfonema magnum*, *Desulfobacterium autotrophicum*, and *Desulfotalea psychrophila* was carried out with a saltwater medium generally used for marine SRB. This medium is characterized by high concentrations of NaCl (20 g/liter) and $MgCl_2 \cdot 6H_2O$ (3 to 5 g/liter). The mineral medium for *Desulfococcus multivorans* contained reduced concentrations of NaCl (7 g/liter) and $MgCl_2 \cdot 6H_2O$ (1.2 g/liter), corresponding to the concentrations in brackish water medium. *Desulfobotulus sapovorans* and *Desulfovibrio vulgaris* were grown in freshwater medium containing the lowest concentrations of NaCl (1 g/liter) and $MgCl_2 \cdot 6H_2O$ (0.4 g/liter). To all mineral media 4 g of Na_2SO_4 per liter and 1 mM sulfide were added. Dithionite (30 mg/liter) was added as an additional reductant prior to inoculation of the media. To support growth of the gliding bacterium *Desulfonema magnum*, insoluble $AlCl_3 \cdot 6H_2O$ was added to the medium as an artificial surface for the gliding movement, as described previously (46). Organic substrates were added from sterile stock solutions. The final concentrations of organic substrates were as follows: lactate, 10, 20, or 40 mM; propionate, 15 mM; caproate, 15 mM; valerate, 10 mM; and benzoate, 3 or 4 mM. Most cultures were incubated at 28°C; *Desulfotalea psychrophila* was incubated at 10°C. The techniques used for preparation of media and for cultivation under strictly anoxic conditions were the techniques described previously (45).

Prior to PHA analysis, cultures of SRB were adapted to the appropriate growth substrates for at least five passages. To obtain sufficient cell mass (1 to 2 g, wet weight) for PHA analysis, about 4 liters of a culture was harvested at the beginning of the stationary growth phase. Because of their fragile cell envelopes, cells of *Desulfonema magnum* were harvested by centrifugation at low speed (200 rpm; JA10 rotor; J2-MC centrifuge; Beckmann). Cells were then washed twice with Tris-HCl (100 mM, pH 7.5), rapidly frozen in liquid nitrogen, and stored at −80°C until analysis.

Cells of *Ralstonia eutropha* PHB$^-$4 and *Pseudomonas putida* GPp104 and cells of recombinant strains of these bacteria were cultivated at 30°C in a mineral salts medium (MSM) described by Schlegel et al. (34). MSM was supplemented with 1.5% (wt/wt) sodium gluconate as the sole carbon source; antibiotics were added if appropriate, as indicated below.

Cells of *Escherichia coli* strains and their recombinants were grown at 37°C in Luria-Bertani (LB) medium (33) with no antibiotics or with antibiotics and with shaking at 150 rpm. Thiamine (50 μM) and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM) were added if *E. coli* harboring pBluescriptSK$^-$ (pSK$^-$) or pBBR1-MCS2 vectors was cultivated. For recombinant strains of *E. coli* harbor-

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ing *pha* genes, PHA accumulation experiments were carried out in liquid LB medium containing 1% (wt/vol) glucose, 0.2% (wt/vol) levulinic acid, or 0.2% (wt/vol) sodium octanoate as the sole carbon source; in some experiments acrylic acid, an inhibitor of fatty acid β-oxidation, was added at a concentration of 0.15 mg/ml when the optical density at 600 nm of the cell suspension was about 1.5 to promote PHA accumulation (28).

Analysis of PHAs. PHAs were analyzed in whole-cell samples or after extraction with chloroform and purification by repeated precipitation from a chloroform solution with ethanol (38). The PHA content and composition were determined by subjecting 5 to 8 mg of lyophilized cells and 1 to 2 mg of isolated PHAs, respectively, to methanolysis, which was done in a mixture of chloroform and methanol containing 15% (vol/vol) sulfuric acid (38). The resulting hydroxyacyl methylesters were analyzed with a Hewlett-Packard type GC6850 gas chromatograph (3, 42). The initial structural assignments of the methylesters analyzed were based on their retention times compared to those of authentic standards. Final confirmation of structures was performed by gas chromatography (GC)-mass spectrometry (MS) by using a model GC6890 gas chromatograph coupled to a model 5973 mass selective detector (Hewlett-Packard).

Preparation of soluble protein fractions from cells. To obtain soluble protein fractions from strains of *E. coli*, cells were grown with shaking in 200 ml of LB medium containing 1% (wt/wt) glucose plus 50 μg of kanamycin per ml or 100 μg of ampicillin per ml at 37°C. IPTG (0.2 mM) was added after an optical density at 600 nm of 0.6 was reached, and cultivation was continued for additional 4 h. Cells of *R. eutropha* or *P. putida* were cultivated in MSM containing 1.5% (wt/vol) sodium gluconate and NH_4Cl at a reduced concentration (0.05%, wt/vol). Cell extracts were obtained by passage through a French pressure cell and centrifugation at 20,000 × *g* at 4°C (10). Protein concentrations were adjusted to 2 mg ml^{-1} before enzyme activity was measured.

Analysis of PHA synthase activity. PHA synthase activity was measured by a spectrometric assay (44). The analysis was done at 30°C in a 500-μl assay mixture consisting of 25 mM Tris-HCl (pH 7.4), 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 20 mM $MgCl_2$, 100 μM D-(−)-3-hydroxybutyryl-CoA, and approximately 30 μg of protein from the soluble protein fraction. Protein was assayed as described by Bradford (2).

Isolation of genomic DNA. For isolation of genomic DNA from SRB we started with about 3 g (wet weight) of cells, and the procedure included resuspension of cells in lysis buffer, addition of sodium dodecyl sulfate, incubation at an elevated temperature, extraction with chloroform-isoamyl alcohol, precipitation with acetate–2-propanol, and washing with ethanol, essentially as described previously (29). Isolated DNA from SRB was completely or partially digested with various restriction endonucleases (EcoRI, EcoRV, BamHI, NdeI, ClaI, PstI, or HindIII) at 37°C for further analysis or for cloning.

Manipulation of DNA, DNA transfer, and other standard techniques. For manipulation of DNA molecules we used standard procedures described by Sambrook et al. (33) or by the enzyme supplier. DNA was transferred to *E. coli* by transformation by using competent cells obtained by the $CaCl_2$ method (33) or to strains of *R. eutropha* and *P. putida* by conjugation by using the spot agar mating technique (8). Analysis of nucleic acids by electrophoresis was done in agarose gels as described by Sambrook et al. (33).

Screening for *pha* genes by using Nile red and the viable colony staining method. To obtain a genomic library of *Desulfococcus multivorans*, genomic DNA of this organism was partially digested with EcoRV and ligated to EcoRV-linearized pHC79 cosmid DNA. The ligation products were packaged into λ-coat proteins by using an in vitro packaging kit (Boehringer, Mannheim, Germany) and were transduced into *E. coli* strain S17-1 by using standard methods (33). About 1,000 transductants were selected and transferred onto LB medium containing ampicillin (100 μg/ml). From *E. coli* S17-1 the genomic library was mobilized into the PHA-negative mutants $PHB^{-}4$ of *R. eutropha* and GPp104 of *P. putida* by spot agar mating (8) in order to identify hybrid cosmids conferring PHA biosynthesis and accumulation to the mutants, thus restoring the phenotype of the wild type. For this, the recombinant mutants were transferred onto MSM agar plates containing 1.5% (wt/vol) sodium gluconate plus kanamycin (150 mg/liter) or Nile red (0.5 μg/ml) to screen for fluorescent recombinants containing PHAs (37).

Screening for *phaC* genes by using PCR and degenerate primers. To screen for *phaC* genes in the genomes of SRB, oligonucleotides were deduced from highly conserved amino acid sequences (VNRPYM and MEKWIF) of known class III PHA synthases and employed as primers in PCR. P1 and P2 (Table 2) were used as forward and reverse primers, and the following steps were used: one cycle at 95°C for 2 min (denaturing), followed by 30 cycles of 42°C for 30 s (annealing), 68°C for 35 s (elongation), and 95°C for 30 s (denaturing). For all PCRs a Platinum *Pfx* DNA polymerase kit (Invitrogen Life Technologies, Carlsbad, Calif.) was employed. DNA of SRB was used as a template after partial restriction with ClaI.

TABLE 2. Oligonucleotides used in this study as primers for PCRs

Primer	5′→3′ sequence[a]
P1	ATNGA(CT)TGGGGNTA(CT) CCN
P2	(AG)AA(AGT)ATCCA(CT)TT(CT)TCCAT
P3 (forward)	CCGCATGGAGAAATGGAT
P4 (reverse)	TCGATGGTGAGGTAGCGAT
P5 (sense)	GGATCGATATGAACCAGCCGAA
P6 (antisense)	GATTCAGATGGTGAGGTA GCGA
P7 (forward)	ACCACGTGAACGGCTACATG
P8 (reverse)	CTTCTTCAGGATCAAATCGA
P9 (sense)	GGACTGGATCCGTCGTATTT
P10 (antisense)	TGACGGATATCATCGCTACC

[a] Sequences representing restriction sites for BamHI (in P9) or EcoRV (in P10) are underlined.

iPCR. Inverse PCR (iPCR) was used to obtain the complete *phaC* genes and adjacent regions (43). To do this, total genomic DNA of *Desulfococcus multivorans* was digested separately with BamHI, EcoRV, ClaI, HindIII, or HincII. The restricted DNA fragments were purified by ethanol precipitation as described by Sambrook et al. (33) before they were ligated to cyclic DNA molecules by employing T4 DNA ligase (MBI Fermentas GmbH, St. Leon-Rot, Germany). They were then incubated with primers P3 (forward) and P4 (reverse) or with primers P7 (forward) and P8 (reverse) (Table 2). The following steps were used: after one cycle at 95°C for 2 min (denaturing), 30 cycles of 54°C for 30 s (annealing), 68°C for 3 min (elongation), and 95°C for 30 s (denaturing). Whereas ClaI-digested genomic DNA gave a 750-bp iPCR product, HindIII- and HincII-digested genomic DNA gave 1,080- and 2,400-bp products, respectively. The iPCR products were purified by using a NucleoTrap kit (Macherey-Nagel GmbH, Düren, Germany) and were ligated to EcoRV-treated pSK^{-} DNA. The ligated products were transformed in *E. coli* TOP10. The nucleotide sequences of the *Desulfococcus multivorans phaE* and *phaC* genes were obtained from the 2,400-bp HincII restriction fragment.

Cloning of *phaC* and *phaEC* from the SRB *Desulfococcus multivorans*. For cloning of *phaC* and *phaEC* from *Desulfococcus multivorans* ($phaC_{Dm}$ and $phaE_{Dm}C_{Dm}$, respectively) PCRs were done by using primers P5 (sense) and P6 (antisense) or primers P9 (sense) and P10 (antisense) (Table 2). The latter primers contained restriction sites for BamHI and EcoRV. These primers were designed on the basis of the nucleotide sequences of $phaC_{Dm}$ and $phaE_{Dm}$ obtained from the HindIII and HincII iPCR products. The PCR cycles included the following steps: after 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 80 s. PCR products that were about 1,200 bp long were purified with a NucleoTrap kit (Macherey-Nagel GmbH) and were then ligated to EcoRV-restricted pSK^{-} and pBBR1-MCS2 DNA by employing T4 DNA ligase (MBI Fermentas GmbH). The hybrid plasmids obtained were then transformed into *E. coli* strains TOP10 and S17-1, respectively, by using standard methods (33), and recombinant strains of *E. coli* harboring pSK::$phaC_{Dm}$ and pBBR-MCS2::$phaC_{Dm}$, respectively, or pSK::$phaE_{Dm}C_{Dm}$ and pBBR1-MCS2::$phaE_{Dm}C_{Dm}$, respectively, were selected (Table 1).

Construction of plasmids expressing hybrid PHA synthases. To express a class III hybrid PHA synthase consisting of the PhaC subunit of *Desulfococcus multivorans* ($PhaC_{Dm}$) and the PhaE subunit of *Thiococcus pfennigii* (formerly *Thiocapsa pfennigii*) ($PhaE_{Tp}$), hybrid plasmid pSK::$phaE_{Tp}C_{Dm}$ was constructed. To do this, a 1.2-kbp DNA fragment containing $phaE_{Tp}$ was obtained from pSelect::B28RV (21) and ligated into XbaI- and EcoRI-restricted pSK::$phaC_{Dm}$ DNA. The resulting hybrid plasmid was transformed into *E. coli* strain TOP10. Similarly, hybrid plasmid pSK::$phaE_{Syn}C_{Dm}$ was constructed, which expressed the PhaE subunit of *Synechocystis* sp. strain PCC6803 obtained in a previous study (10) and $PhaC_{Dm}$.

DNA sequence analysis and alignment. For DNA sequencing the dideoxy chain termination method was used. Infrared dye-labeled primers (IRD750 5′ end modification) from MWG Biotech AG (Ebersberg, Germany), a Sequitherm Excel Long DNA sequencing kit (LC Epicentre Technologies, Madison, Wis.), and a LI-COR sequencer (LI-COR, Lincoln, Nebr.) were used. Nucleotide and deduced amino acid sequences were analyzed with Heidelberg UNIX Sequence Analysis Resources (HUSAR), release 4.0, and the Wisconsin program package (Unix-8.1, 1995). A homology search was performed by performing a BLAST search of the National Center for Biotechnology Information database. For alignment of nucleotide sequences, as well as alignment of deduced amino acid

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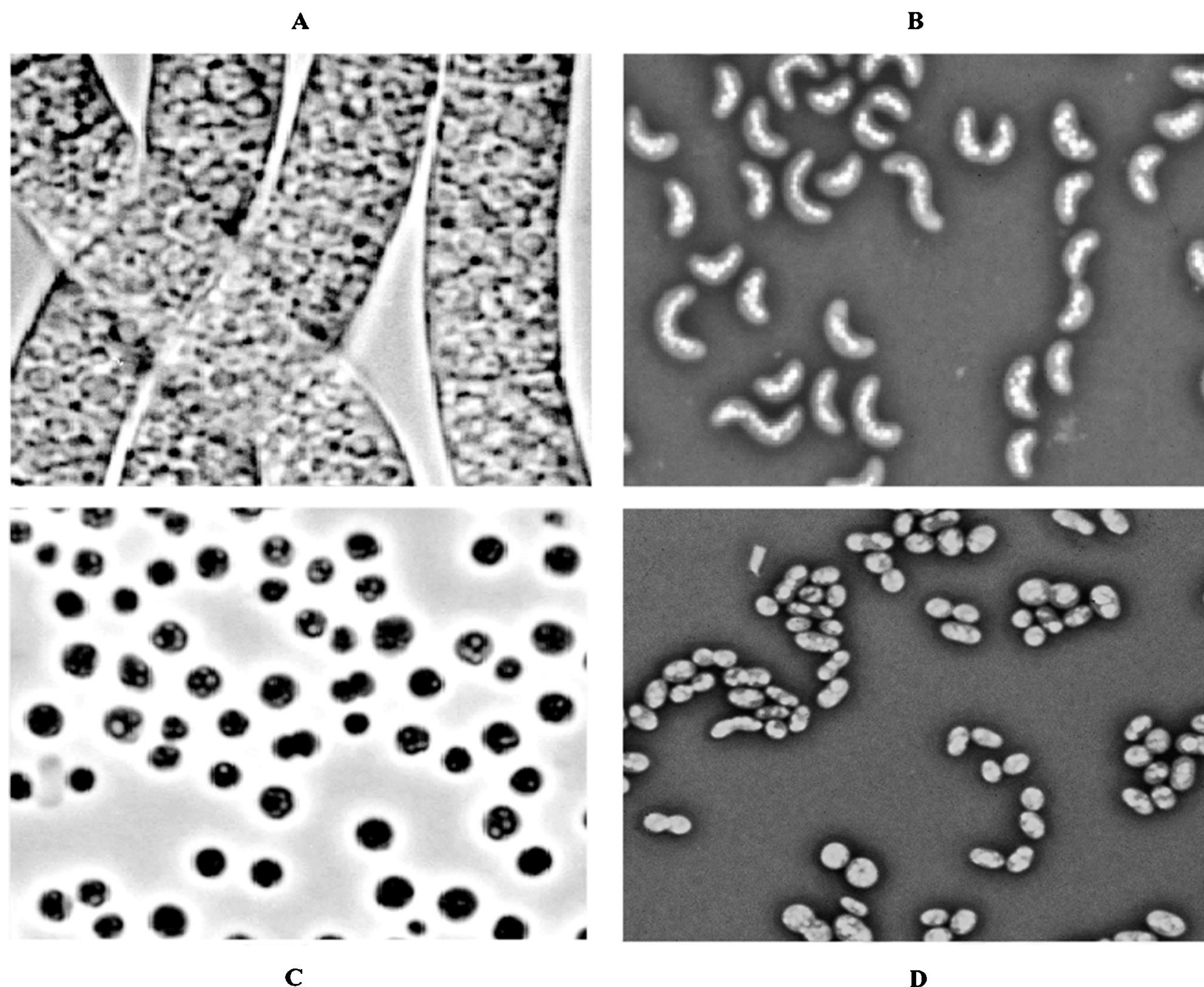

FIG. 1. Light microscopic images of SRB accumulating large amounts of PHA. (A) *Desulfonema magnum*; (B) *Desulfobotulus sapovorans*; (C) *Desulfococcus multivorans*; (D) *Desulfosarcina variabilis*. Bar = 10 μm.

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sequences and construction of phylogenetic trees for 16S rRNA genes, the PHYLIP program package with global rearrangement was used (7).

Preliminary sequence data for the *Desulfobacterium autotrophicum* and *Desulfovibrio vulgaris* genomes were obtained from the REGX Consortium (www.regx.de) and The Institute for Genomic Research website (http://www.tigr.org).

Nucleotide sequence accession number. The DNA sequences of *phaE* and *phaC* of *Desulfococcus multivorans* have been deposited in the EMBL database under accession number AY363615.

RESULTS AND DISCUSSION

PHA accumulation in SRB. Seven species belonging to different genera of SRB were analyzed to determine their abilities to synthesize PHAs. In the species investigated, the highest PHA content (about 90% [wt/wt] of cell dry matter) was measured for cells of benzoate-grown *Desulfonema magnum*. The fragility of the large *Desulfonema magnum* cells resulted in partial destruction during harvesting, even though centrifugation was carried out at a low speed. Therefore, the PHA contents of *Desulfonema magnum* samples analyzed by GC might have been slightly overestimated. Nevertheless, phase-contrast microscopic images impressively revealed the large number of closely packed inclusions in the cytoplasm, which also indicated that the PHA content of the cells was very high (Fig. 1). GC analysis of benzoate-grown cells of *Desulfococcus multivorans* and *Desulfosarcina variabilis* and of caproate-grown cells of *Desulfobotulus sapovorans* revealed that the hydroxyalkanoic acid contents were about 27, 22, and 43% (wt/wt) of the cell dry matter, respectively. If cells of *Desulfobacterium autotrophicum* were cultivated with valerate or caproate, the hydroxyalkanoic acid contents were about 8 and 11% (wt/wt) of the cell dry matter, respectively, whereas in cells cultivated with lactate PHAs could not be detected. *Desulfotalea psychrophila* and *Desulfovibrio vulgaris* (both lactate grown) were the only SRB for which no evidence of the presence of PHAs was obtained. In most hydroxyalkanoic acid-positive samples GC and GC-MS analyses revealed that 3-hydroxybutyrate (3HB) was by far the major compound.

Analysis of whole-cell samples also revealed the presence of some other hydroxyalkanoic acids, such as 3-hydroxyvaleric

TABLE 3. Chemical compositions of PHAs purified from cells of SRB

Species	Carbon source[a]	Concn of isolated PHAs (% [wt/wt] of cell dry matter)[b]	Composition of PHAs (%, wt/wt)[c]	
			3HB	3-Hydroxyvaleric acid
Desulfococcus multivorans	Benzoate	8.0	100	ND
Desulfobotulus sapovorans	Valerate	9.8	49	51
Desulfobotulus sapovorans	Caproate	13.5	100	ND
Desulfonema magnum	Benzoate	88.0	100	ND
Desulfobacterium autotrophicum	Valerate	5.4	48	52

[a] Mineral medium containing 0.025% (wt/vol) NH_4Cl as described in Materials and Methods was used as the basic medium and was supplemented with the carbon sources indicated.
[b] PHAs were extracted from the cells with chloroform, precipitated with ethanol, and subsequently dried. The amount of isolated PHAs as determined by GC analysis was compared with the amount of cells used as the starting material for extraction.
[c] The amounts of comonomers of the isolated PHAs were analyzed by GC. The structures of the constituents detected were confirmed by GC-MS. The experiments were carried out in triplicate and were performed by using the protocols described in Materials and Methods. ND, not detectable (detection limit, about 1% [wt/wt]).

acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, and 3-hydroxytetradecanoic acid, and of fatty acids, such as palmitic acid. Since they occurred at low concentrations and could also be derived from other cell constituents, such as phospholipids or triacylglycerols, PHAs were isolated from some cells and analyzed (Table 3). GC and GC-MS analyses of the isolated and purified PHAs clearly revealed that cells of *Desulfococcus multivorans*, *Desulfobotulus sapovorans*, and *Desulfonema magnum* accumulated the poly(3HB) homopolyester. Only valerate-grown cells of *Desulfobotulus sapovorans* and *Desulfobacterium autotrophicum* accumulated copolymers of 3HB and 3-hydroxyvaleric acid containing almost equimolar amounts of the comonomers (Table 3). We therefore concluded that PHA-accumulating SRB are of the PHA_{SCL} type.

Screening for *pha* genes based on heterologous expression. Our first screening of genomic libraries prepared from BamHI- or EcoRV-restricted *Desulfococcus multivorans* genomic DNA and BamHI- or EcoRV-restricted cosmid pHC79 DNA in *E. coli* strain S17-1 did not reveal clones that formed opaque colonies on LB-glucose agar plates, indicating that none of the clones analyzed contained DNA fragments conferring PHA_{SCL} biosynthesis to *E. coli*. The libraries were subsequently also transferred from *E. coli* S17-1 to the PHA-negative mutant $PHB^{-}4$ of *R. eutropha* by conjugation and screened on MSM-gluconate agar plates containing Nile red. However, only a few clones exhibited very low fluorescence; the cells of these clones contained barely more PHA than the negative control contained, as determined by GC. It was therefore obviously not possible to identify the *Desulfococcus multivorans* PHA biosynthesis genes by conferring PHA biosynthesis to PHA-negative strains, as was successfully done in the past with corresponding genes from many other bacteria (31).

Screening for *pha* genes by using PCR techniques. The use of ClaI-restricted genomic DNA from *Desulfococcus multivorans* and of primers P1 and P2, which were designed based on highly conserved regions of PHA synthases, yielded an approximately 500-bp PCR product. This PCR product was ligated to EcoRV-linearized pSK^{-} DNA, transformed into *E. coli* TOP10, and sequenced, revealing a 536-bp fragment (P536) (Fig. 2). A comparison of the amino acid sequence deduced from P536 by using the BLAST network service programs resulted in up to 35% identity for 175 amino acids of known PhaC subunits of class III PHA synthases, thus indicating that a central region of the *Desulfococcus multivorans* PHA synthase structural gene ($phaC_{Dm}$) was obtained.

For identification of the entire $phaC_{Dm}$ gene by iPCR, oligonucleotides P3 and P4, hybridizing to the 3′ and 5′ regions of P536, respectively, were used as primers, and ClaI- or HindIII-restricted and religated genomic DNA from *Desulfococcus multivorans* was used as the template (Fig. 2). ClaI-restricted template DNA yielded a 750-bp iPCR product, and HindIII-restricted template DNA yielded a 1,080-bp iPCR product. The iPCR products were ligated to EcoRV-restricted pSK^{-} DNA and then transformed into *E. coli* TOP10 and subse-

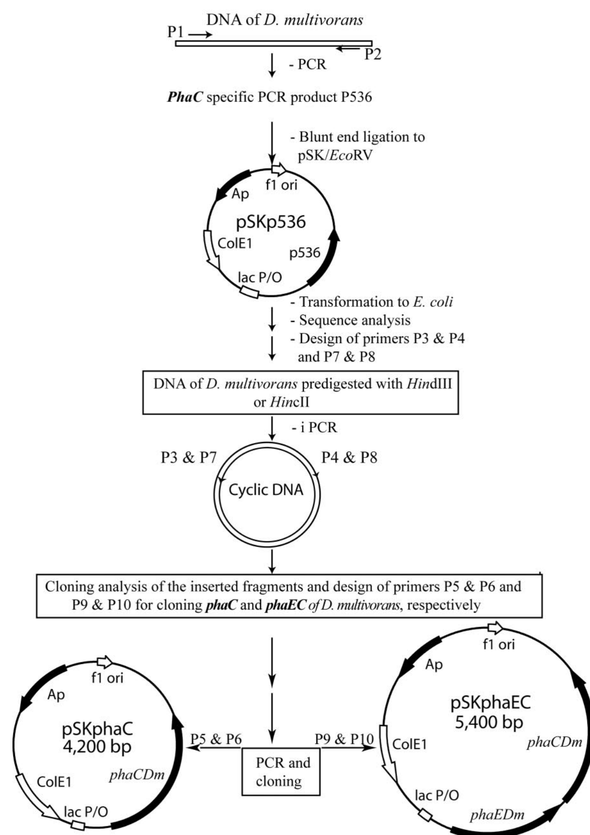

FIG. 2. Cloning and organization of the *Desulfococcus multivorans pha* locus. The flow diagram describes the procedure used to clone *phaC* and *phaE* from *Desulfococcus multivorans* by PCR and iPCR techniques. The organization of the two genes, relevant cleavage sites of restriction endonucleases, DNA fragments, and locations of primers are shown. Primer sequences are shown in Table 2.

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TABLE 4. Similarities and differences among amino acid sequences of known or putative PhaC and PhaE subunits of class III PHA synthases to $PhaC_{Dm}$ and $PhaE_{Dm}$

Species or strain	PhaC			PhaE			Accession no.	
	No. of amino acids	% Similarity[a]	% Identity[b]	No. of amino acids	% Similarity[a]	% Identity[b]	*phaC*	*phaE*
Desulfococcus multivorans	371	100	100	306	100	100	—[e]	—
T. violacea	355	62	45	364	37	22	D48376	S54369.1
Allochromatium vinosum	355	61	45	357	43	23	S29274	P45372
Xanthomonas axonopodis strain 306	358	63	43	407	42	22	AE011840.1	NC003919.1
Xanthomonas campestris ATCC 33913	358	62	43	387	41	20	AE012323.1	NC003902.1
Ectothiorhodospira shaposhnikovii	355	65	44	371	44	19	AF307334.1	AF307334.1
Synechocystis sp. strain PCC6803	378	62	44	330	37	20	D90906.1	S77326
Synechococcus sp. strain MA19	364	65	45	NK[d]	NK	NK	AY030295.1	
Chlorogloeopsis fritschii strain PCC6912	366	64	44	NK	NK	NK	AF3713691	
Magnetococcus sp. strain MC-1	353	65	49	425	32	19	NZ AAAN01000071.1	NZ AAAN01000071.1
Bacillus megaterium[c]	362	56	33	199	49	24	AF109909.2	AF109909.2

[a] The level of similarity was determined by pairwise comparison to *D. multivorans* genes by using the tblastX software.
[b] The level of identical amino acids was determined in comparison to *D. multivorans* genes by a BLAST search using the tblastX software.
[c] The amino acid sequence of PhaR of *B. megaterium* was used in the homology search.
[d] NK, not known.
[e] —, sequence determined in this study.

quently sequenced. Whereas the 750-bp iPCR product revealed about 300 bp of the upstream region and about 400 bp of the downstream region of P536, sequence analysis of the 1,080-bp iPCR product gave the complete sequence of $phaC_{Dm}$, which comprised 1,116 bp.

Primers P7 and P8 were designed from $phaC_{Dm}$ and used for a second iPCR, in which HincII-restricted and religated genomic DNA of *Desulfococcus multivorans* was used to obtain the regions adjacent to $phaC_{Dm}$ (Fig. 2). One approximately 2.4-kbp iPCR product was obtained and sequenced. Together with the known sequence of $phaC_{Dm}$, a 2,877-bp sequence was obtained. This sequence comprised a complete *phaE* homologous gene upstream of $phaC_{Dm}$. No other homologous genes related to PHA metabolism were detected adjacent to $phaE_{Dm}$ or $phaC_{Dm}$. The G+C content of the $phaE_{Dm}C_{Dm}$ sequence was 54.5%, which corresponded to the G+C content of the 16S ribosomal DNA gene of *Desulfococcus multivorans* (54.0%) (5). For further analysis a 2,450-bp fragment containing $phaE_{Dm}C_{Dm}$ was cloned.

Characteristics of the $PhaC_{Dm}$ subunit of the PHA synthase. $phaC_{Dm}$ comprises 1,116 bp encoding a predicted protein containing 371 amino acids. The amino acid sequence exhibited strong homology to the sequences of confirmed or putative PhaC subunits of class III PHA synthases from other bacteria (33 to 49% identical amino acids) (Table 4). In contrast, when $PhaC_{Dm}$ was compared with PhaC subunits of class I and class II PHA synthases, the maximal amino acid identities were only 30 and 25%, respectively.

The amino acid sequence deduced from $phaC_{Dm}$ also contained highly conserved regions adjacent to amino acids C149, D302, H303, and H331 of the PhaC sequence of *Allochromatium vinosum* (20). These residues are involved in activation of

TABLE 5. PHA accumulation and in vitro PHA synthase activities of the recombinant strains of *E. coli*, *R. eutropha* PHB^-4, and *P. putida* GPp104[a]

Strain	Plasmid(s)	Medium[b]	PHA content (%, wt/wt)[c]	Sp act (U mg of protein^{-1})[d]
E. coli strains				
S17-1	pBBR1-MCS2	LB	ND	ND
TOP10	pSK^-	LB	ND	ND
TOP10	pSK^-::$phaC_{Dm}$	LB + IPTG	ND	0.1
TOP10	pSK^-::$phaA_{Syn}B_{Syn}$	LB + IPTG	0.5	ND
S17-1	pBBR1-MCS2::$phaC_{Dm}$	LB + IPTG	ND	0.2
TOP10	pSK^-::$phaE_{Syn}C_{Dm}$	LB + IPTG	ND	1.5
TOP10	pSK^-::$phaE_{Tp}C_{Dm}$	LB + IPTG	ND	0.2
TOP10	pBBR1-MCS2::$phaEC_{Dm}$	LB + IPTG	1.7	1.0
S17-1	pSK^-::$phaA_{Syn}B_{Syn}$, $pBBR1\text{-}MCS2^-$::$phaA_{Syn}B_{Syn}$	LB + IPTG	Trace	0.1
S17-1	pSK^-::$phaE_{Tp}C_{Dm}$, $pBBR1\text{-}MCS2^-$::$phaA_{Syn}B_{Syn}$	LB + IPTG	Trace	ND
P. putida GPp104	pSK::$phaC_{Dm}$, pBBR1-MCS2::$phaC_{Dm}$	MSM	1.0	0.4
R. eutropha PHB^-4	pBBR1-MCS2::$phaC_{Dm}$	MSM	1.5	0.5
P. putida GPp104	pBBR1-MCS2::$phaE_{Dm}C_{Dm}$	MSM	0.7	0.5
R. eutropha PHB^-4	pBBR1-MCS2::$phaE_{Dm}C_{Dm}$	MSM	1.2	1.6

[a] PHA analysis and enzyme activity measurement were carried out in triplicate and were performed by using the protocols described in Materials and Methods.
[b] IPTG was added at a final concentration of 0.2 mM. For PHA accumulation studies, LB medium was supplemented with 1% (wt/vol) glucose (for *E. coli*) and MSM was supplemented with 1.5% (wt/vol) sodium gluconate (for *R. eutropha* PHB^-4 and *P. putida* GPp104).
[c] The amount of isolated PHA as determined by GC analysis was compared with the amount of cells used for analysis. ND, not detectable (detection limit, less than 0.1% [wt/wt]).
[d] One unit of enzyme activity was defined as the activity that converted 1 μmol of substrate per min. ND, not detectable (detection limit, less than 0.01 U/mg of protein).

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the 3-hydroxyl alkyl moiety of 3-hydroxybutyryl-CoA (D302, H303), support nucleophilic attack (H331), and are involved in covalent catalysis (C149) (14). PhaC_{Dm} possesses the highly conserved motif RMEKWIFDSPD (amino acid residues 245 to 254), which is typical for a class III synthase. In contrast, the highly conserved cysteine residue at position 130 in the sequence of *A. vinosum*, which is considered to be important for the *A. vinosum* PHA synthase to exhibit enzyme activity (27), was replaced by a valine residue in PhaC_{Dm}. In addition, PhaC_{Dm} does not contain the cyanobacterial box (RGGCTL GAEA), which was identified as a characteristic feature of PhaC from cyanobacteria (10).

Characteristics of the PhaE_{Dm} subunit of the PHA synthase. *pha*E_{Dm} comprises 921 bp encoding a predicted protein consisting of 306 amino acids. The amino acid sequence exhibited homology to the sequences of confirmed or putative PhaE subunits of class III PHA synthases from other bacteria. However, the levels of similarity were generally much lower, and the levels of identical amino acids varied between 19 and 23%. In addition, more gaps were present, indicating the presence of highly variable regions in the PhaE proteins. The levels of amino acid identity between PhaE_{Dm} and the most closely related PhaE subunits from other bacteria ranged from 19 to 23% (Table 4). Besides these PhaE subunits, homology was observed only with PhaR of *Bacillus megaterium* (24% amino acid identity), which is the second subunit of the class IV PHA synthase of this bacterium (25).

Most PhaE subunits possess three distinguishable domains, which are referred to as domains I, II, and III. The amino acid stretch FMRSPLLGPSR from *Synechocystis* sp. strain PCC6803 (domain I), corresponding to amino acid positions 146 to 156 in PhaE_{Dm}, is the most highly conserved region. Domain II, which was recently referred to as the PhaE box (10), is also present in PhaE_{Dm} and comprises amino acid positions 267 to 271. Domain III, which was suggested as a probable granule binding site of some class III PHA synthases (21), is most probably absent in PhaE_{Dm} because the corresponding stretch KLDNRLSEEPA (amino acid positions 292 to 302) contains too many negatively charged and polar amino acids.

Heterologous expression of the *Desulfococcus multivorans* PHA synthase in *E. coli*. DNA fragments comprising only *pha*C_{Dm} and only *pha*$E_{Dm}C_{Dm}$ were obtained by PCR by using primers P5 and P6 and primers P9 and P10, respectively. These PCR products were inserted into the EcoRV restriction sites of plasmids pSK^- and pBBR1-MCS2 and transformed into *E. coli* strain TOP10 or S17-1, and the cells of the recombinant strains were analyzed for PHA synthase activity and PHA content (Table 5).

No enzyme activity and no PHA accumulation were observed with cells harboring only the vector. Cells harboring only *pha*C_{Dm} had very low PHA synthase activity and did not accumulate any detectable PHA. Cells harboring *pha*C_{Dm} plus *pha*E_{Dm} had significantly higher PHA synthase activity; however, these cells also did not accumulate any detectable PHA. This was due to the lack of 3HB-CoA biosynthesis. If *E. coli* harbored a second plasmid encoding PhaA and PhaB of *Synechocystis* sp. strain PCC6308 to ensure provision of 3-hydroxybutyryl-CoA to the PHA synthase, poly(3HB) was synthesized and accumulated.

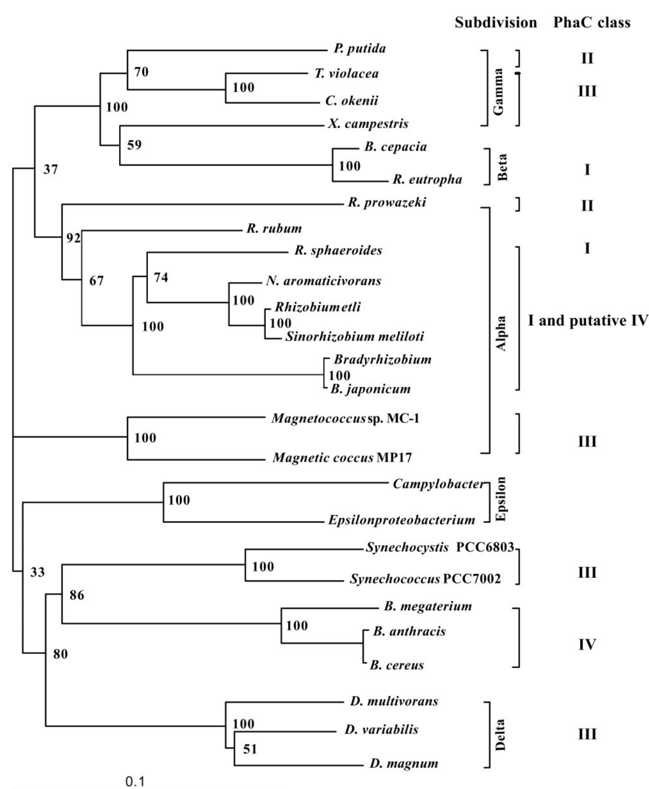

FIG. 3. Occurrence of class III PHA synthases in eubacteria. The phylogenetic tree, based on 16S rRNA sequences for selected taxa of eubacteria, was obtained by using the neighbor-joining algorithm. The numbers next to the nodes indicate the bootstrap values based on 100 resamplings (expressed as percentages). Scale bar = 0.1 substitution per site. The evolutionary distances for the rRNA genes were reconstructed by using a multiple alignment of 1,320 nucleotide sequence positions, including the sequences of variable regions. The distribution of classes of PHA synthases in different taxa of eubacteria, including the alpha, beta, gamma, delta, and epsilon subdivisions of the *Proteobacteria*, is indicated on the right. *T. violacea*, *Thiocystis violacea*; *C. okenii*, *Chromatium okenii*; *B. cepacia*, *Burkholderia cepacia*; *R. prowazeki*, *Rickettsia prowazekii*; *R. rubrum*, *Rhodospirillum rubrum*; *R. sphaeroides*, *Rhodobacter sphaeroides*; *B. japonicum*, *Bradyrhizobium japonicum*; *B. anthracis*, *Bacillus anthracis*; *B. cereus*, *Bacillus cereus*.

Heterologous expression of the *Desulfococcus multivorans* PHA synthase in *R. eutropha* and *P. putida*. From *E. coli* S17-1 pBBR1-MCS2::*pha*C_{Dm} and pBBR1-MCS2::*pha*$C_{Dm}E_{Dm}$ were also mobilized to mutants PHB^-4 and GPp104 of *R. eutropha* and *P. putida*, respectively. Analyses of the recombinant strains revealed higher values for PHA synthase activities in crude extracts, as well as poly(3HB) in the cells, if both genes were present (Table 5). We concluded that in *E. coli*, *R. eutropha*, and *P. putida* PhaC_{Dm} confers low but significant PHA synthase activity, particularly if PhaE_{Dm} is also expressed. The low enzyme activities were in accordance with the low PHA contents of the recombinant strains and provide a reasonable explanation for why our attempts to identify clones harboring the *pha* locus of *Desulfococcus multivorans* by an approach based on heterologous expression and phenotypic complementation failed.

Construction of two-component hybrid PHA synthases. Since plasmids containing *phaE* and *phaC* of *Desulfococcus*

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multivorans conferred only low levels of PHA synthase activity and PHA accumulation to other bacteria, plasmids encoding different hybrid PHA synthases, such as pSK$^-$::$phaE_{Tp}C_{Dm}$ (PhaE of *T. pfennigii* plus PhaC_{Dm}), pSK$^-$::$phaE_{Syn}C_{Dm}$, and pBBR1-MCS2::$phaE_{Syn}C_{Dm}$ (PhaE of *Synechocystis* sp. strain PCC6803 plus PhaC_{Dm}) were constructed and transformed into *E. coli*, as was previously done for other class III hybrid PHA synthases (21). Recombinant strains of *E. coli* harboring these plasmids expressed significant but low in vitro PHA synthase activities. However, the activities were not higher than the activities with the homomeric enzymes (Table 5). Provision of 3-hydroxy fatty acid monomers in vivo was achieved by coexpression of pSK$^-$::$phaE_{Syn}C_{Dm}$ with pSK$^-$::$phaA_{Syn}B_{Syn}$ during cultivation of *E. coli* strain S17-1 in LB-glucose medium containing IPTG or by cultivation of *E. coli* TOP10 harboring pSK$^-$::$phaE_{Syn}C_{Dm}$ in LB-glucose medium containing levulinic acid plus acrylic acid as an inhibitor of fatty acid β-oxidation. However, accumulation of PHA in the recombinant cells was low or even absent (only some of the data are shown in Table 5).

Occurrence of class III PHA synthases. The general occurrence of class III PHA synthases in PHA-accumulating SRB is indicated by the detection of genes coding for homologous PhaC and PhaE proteins in *Desulfococcus multivorans* (this study) and in the genome sequence of *Desulfobacterium autotrophicum* (www.regx.de) and by successful amplification of *phaC* fragments by PCR from some other SRB (data not shown). Interestingly, no genes coding for homologous PHA synthase proteins were found in the genome of *Desulfovibrio vulgaris*. This finding is, however, consistent with the lack of detectable PHA in this bacterium. Two-component class III PHA synthases are now known to occur in a wide range of different bacteria and have been detected in anoxygenic purple sulfur bacteria (e.g., *A. vinosum* [19]), in cyanobacteria (e.g., *Synechocystis* sp. strain PCC6803 [11, 17]), in species of the genus *Xanthomonas* (e.g., *Xanthomonas campestris* [4], in marine magnetotactic bacteria (e.g., *Magnetospirillum magnetotacticum* [NCBI Microbial Genomes Annotation project; contig with accession number NZ AAAN01000071]), in *Novosphingobium aromaticivorans* (NCBI Microbial Genomes Annotation project; DOE Joint Genome Institute accession number NZ AAAV01000165.1), and in SRB (this study). A phylogenetic tree which is based on 16S rRNA genes shows that class III PHA synthases do not occur only in a particular group of phylogenetically related bacteria (Fig. 3). The distribution of class III PHA synthases in so many bacteria that are not very closely related phylogenetically is interesting and might indicate that the genes for these enzymes were acquired from a common source by horizontal DNA transfer during evolution. It is unlikely that they evolved independently.

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