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Genetic and Biochemical Characterization of the Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) Synthase in *Haloferax mediterranei*▽†

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The haloarchaeon *Haloferax mediterranei* has shown promise for the economical production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), a desirable bioplastic. However, little is known at present about the genes involved in PHBV synthesis in the domain *Archaea*. In this study, we cloned the gene cluster (*phaEC*$_{Hme}$) encoding a polyhydroxyalkanoate (PHA) synthase in *H. mediterranei* CGMCC 1.2087 via thermal asymmetric interlaced PCR. Western blotting revealed that the *phaE*$_{Hme}$ and *phaC*$_{Hme}$ genes were constitutively expressed, and both the PhaE$_{Hme}$ and PhaC$_{Hme}$ proteins were strongly bound to the PHBV granules. Interestingly, CGMCC 1.2087 could synthesize PHBV in either nutrient-limited medium (supplemented with 1% starch) or nutrient-rich medium, up to 24 or 18% (wt/wt) in shaking flasks. Knockout of the *phaEC*$_{Hme}$ genes in CGMCC 1.2087 led to a complete loss of PHBV synthesis, and only complementation with the *phaEC*$_{Hme}$ genes together (but not either one alone) could restore to this mutant the capability for PHBV accumulation. The known haloarchaeal PhaC subunits are much longer at their C termini than their bacterial counterparts, and the C-terminal extension of PhaC$_{Hme}$ was proven to be indispensable for its function in vivo. Moreover, the mixture of purified PhaE$_{Hme}$/PhaC$_{Hme}$ (1:1) showed significant activity of PHA synthase in vitro. Taken together, our results indicated that a novel member of the class III PHA synthases, composed of PhaC$_{Hme}$ and PhaE$_{Hme}$, accounted for the PHBV synthesis in *H. mediterranei*.

Polyhydroxyalkanoates (PHAs) are a broad class of polyesters of various hydroxyalkanoates that are accumulated as carbon and energy storage materials in many bacteria and archaea under nutrient-limiting conditions with excess carbon source (31). Due to their excellent biodegradability, biocompatibility, and mechanical properties, PHAs have been drawing much attention as promising substitutes for petroleum-derived plastics (30). However, the high production cost of PHAs is still an obstacle to their widespread use, and therefore, much effort should be devoted to bringing down the production costs, such as using inexpensive carbon sources or developing more economical fermentation and separation processes (17, 42). Indeed, several haloarchaeal strains in the genera *Haloferax*, *Halobacterium*, *Haloarcula*, and *Haloquadratum* can synthesize PHAs from cheap carbon sources, and isolation of PHAs from these haloarchaea is much easier than from bacteria (8, 11, 12, 16, 20, 23). Therefore, haloarchaea provide a novel opportunity to produce PHAs economically.

Due to its high growth rate, metabolic versatility, and genetic stability, *Haloferax mediterranei* has become an interesting archaeon for investigating metabolites, including PHAs (3, 8, 14, 23, 26). The PHA accumulated by *H. mediterranei* was reported to be poly(3-hydroxybutyrate) (PHB) originally (8, 23) but has been reevaluated as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) recently (5, 17). PHBV has much better mechanical properties than PHB and hence is more promising for commercial production and application (1, 19). Unlike PHBV production in bacteria, where costly and cellular toxic carbons, such as propionic acid or valeric acid, would be provided as the precursors of the 3-hydroxyvalerate (3HV) unit (36), *H. mediterranei* can accumulate PHBV up to ~60% (wt/wt) from starch, glucose, or other cheaper carbon sources, including industrial by-products (17, 23). Thus, *H. mediterranei* has become one of the most promising candidate organisms for industrial PHA production.

In bacteria, extensive research over several decades has accumulated much information on the pathways of PHA synthesis and degradation (38). PHA synthases, the key enzymes catalyzing the polymerization of 3-hydroxyacyl-coenzyme A (CoA) into PHAs, are generally grouped into four classes in bacteria according to their substrate specificities and the subunit compositions (37). Class III and IV synthases are composed of two subunits; one is PhaC, and the other is PhaE (~40 kDa) (10, 21, 22) or PhaR (~22 kDa) (28). In the domain *Archaea*, however, little was known about the genes and enzymes involved in PHA synthesis until recently, when the first archaeal-type *phaEC* genes encoding a putative class III PHA synthase were identified and characterized in *Haloarcula marismortui* and *Haloarcula hispanica* (11). For PHBV biosynthesis in *H. mediterranei*, although much preliminary work has been performed (17), the molecular and genetic information for PHBV metabolism in this halophilic archaeon remains unknown.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
H. mediterranei CGMCC 1.2087	Wild-type strain = ATCC 33500	CGMCC
H. hispanica ATCC 33960	Wild-type strain	ATCC
H. mediterranei Δ*phaEC*$_{Hme}$	*phaEC*$_{Hme}$-deleted mutant of *H. mediterranei*	This study
H. hispanica PHB-1	*phaEC*$_{Hh}$-deleted mutant of *H. hispanica*	11
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*	35
Plasmids		
pGEM-T	3.0-kb vector for PCR product cloning; Amp^r	Promega
pWL102	10.5-kb shuttle vector; Amp^r Mev^r	18
pWL3E	11.2 kb; *phaE*$_{Hme}$ and its native promoter	This study
pWL3C	12.1 kb; *phaC*$_{Hme}$ and promoter of *phaE*$_{Hme}$	This study
pWL3EC	12.6 kb; *phaEC*$_{Hme}$ and its native promoter	This study
pWL3ECS-1	12.3 kb; 3′-truncated *phaEC*$_{Hme}$ and its native promoter	This study
pUBP	6.6 kb; derivative of pUBP2 by removing the pHH9-*ori* region	11
pLHC	7.9 kb; integration vector for knockout of *phaEC*$_{Hme}$ of *H. mediterranei* CGMCC 1.2087	This study
pWLEhis	11.2 kb; shuttle vector for His-tag-fused PhaE	This study
pWLChis	12.1 kb; shuttle vector for His-tag-fused PhaC	This study
pWLEC	12.6 kb; *phaEC*$_{Hm}$ and its native promoter	11

In the present study, we report for the first time the gene cloning and molecular characterization of the PHBV synthase in *H. mediterranei*. Both genetic and biochemical evidence demonstrated that the PHBV synthase in *H. mediterranei* is actually composed of two subunits, PhaE$_{Hme}$ and PhaC$_{Hme}$. Taking these data together with our previous studies (11) and phylogenetic analysis, we report that the class III PHA synthase is widespread in the domain *Archaea*.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. The strains and plasmids used in this study are listed in Table 1. The oligonucleotides are listed in Table 2. *Escherichia coli* JM109, used as a host for cloning experiments, was grown in Luria-Bertani medium at 37°C (35). When required, ampicillin was added to a final concentration of 100 mg/liter. Generally, *H. mediterranei* and *H. hispanica* strains were cultivated at 37°C in nutrient-rich AS-168 medium (per liter, 200 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 3 g trisodium citrate, 1 g sodium glutamate, 50 mg $FeSO_4 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 5 g Bacto Casamino Acids, 5 g yeast extract, pH 7.2). For PHA accumulation analysis, *H. mediterranei* and *H. hispanica* cells were first grown at 37°C for 48 h in AS-168 medium, and then 5% inocula were transferred to 100 ml nutrient-limited MST (per liter, 200 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 1 g sodium glutamate, 37.5 mg KH_2PO_4, 50 mg $FeSO_4 \cdot 7H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 1 g yeast extract, 10 g starch, pH 7.2) or MG medium (the same composition as MST except for glucose as a substitute for starch) in shaking flasks and cultivated for an additional 72 h. The pH was manually adjusted to be maintained at about 7.2 in the media. The haloarchaeal expression plasmids derived from pWL102 (Table 1) were usually first constructed in *E. coli* JM109 and then transformed into *H. mediterranei* or *H. hispanica* by a polyethylene glycol-mediated transformation method (4). When needed, mevinolin was added to a final concentration of 3 mg/liter or 5 mg/liter for *H. mediterranei* or *H. hispanica* transformants.

Preparation and analysis of PHA granules. The cellular PHA content and its composition were analyzed by gas chromatography (GC) (11). Briefly, 70 to 80 mg of lyophilized haloarchaeal cells was subjected to methanolysis in a mixture of chloroform and methanol containing 3% (vol/vol) sulfuric acid at 100°C for 4 h. The resulting hydroxyacyl methylesters were then analyzed with Agilent GC-6820. PHBV (Sigma) was used as the standard sample, with benzoic acid as the inner standard. To obtain PHA granules, *H. mediterranei* cells cultivated in MST medium were harvested by centrifugation, washed, and resuspended in TBS buffer (per liter, 20 mmol Tris-HCl [pH 7.5], 200 g NaCl, 20 g KCl, 5 g $MgSO_4 \cdot 7H_2O$). Crude extracts were prepared by ultrasonic treatment of these cells, and the intact cells and debris were pelleted by centrifugation (15 min; 8,000 × *g*; 4°C). The PHA granules were purified by subsequent ultracentrifugation as described previously (11).

TEM analysis. *H. mediterranei* cells cultivated in AS-168 or MST medium for 48 h were harvested by centrifugation and then subjected to transmission electron microscopy (TEM) analysis. Briefly, the cells were washed twice with sodium phosphate buffer (10% NaCl, 0.1 M sodium phosphate buffer [pH 7.2]) and then suspended in a solution (2.5% [vol/vol] glutaraldehyde, 10% NaCl, and 0.1 M sodium phosphate buffer [pH 7.2]) for primary fixation. After 2 h of fixation,

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

Primer	5′–3′ sequence[a]
P1	CTCATCGTTTACGCGCTCATCAAC
P2	GGCCTCCGGCGGGATGAGGTGGTC
AD1	NGTCGASWGANAWGAA
AD2	AGWGNAGWANCAWAGG
AD3	NTCGASTWTSGWGTT
dp1	TACGTCCGGTTGTTCGAG
dp2	GCAGTTCTTGGAGGACGT
dp3	CCAGATTGTCGGGGAGTA
up1	CCATGCAGTACCCGAGAA
up2	CGTGAGGTGGGTGTCGAG
up3	GGTTGGAGGTCGAGAATG
KF1	AGCCTGCAGCGGGTACGGGTGTTTCAG
KR1	ATAGGATCCACAGACTACTCCGGCGTG
KF2	ATAGGATCCGGCGAGTAAACCGTTCAA
KR2	ATAGGTACCATCGGGAGCTGTACGGAC
phaEF1	AGACGCCATGGACGAAAT
phaER1	ATAGGATCCTCATCCCTCCACGTCCAT
phaCR1	ATAGGATCCATCGGGAGCTGTACGGAC
phaCF1	ATAGGATCCATGACACCAGTAACCT TCGC
phaCR2	TCGGAGTTCCAGGTACCA
PER	ATAGGATCCGGGCATATACTCTCGGGC
phaCRS1	ATAGGATCCTTATGAACGTTCGGCGA ACCA
phaEFH	CCGACATATGTCACAACAAAAAG GGGA
phaERH	TTAGGTACCTTAGTGGTGGTGGTGGTG GTGTCCCTCCACGTCCATCGC
phaCFH	CCGACATATGACACCAGTAACCTTCGC
phaCRH	TTAGGTACCTTAGTGGTGGTGGTGGTG GTGGTCGGTGCGGTCGGATGC
PL	CGGGTACGGGTGTTTCAG
PR	ATCGGGAGCTGTACGGAC

[a] Sequences representing restriction sites are underlined.

the cells were pelleted and washed with sodium phosphate buffer three times. The cell pellets were then used for secondary fixation with 1.0% osmium tetroxide (OsO_4) in 10% NaCl overnight. After three washes with sodium phosphate buffer, TEM analysis was performed according to the protocol described by Tian et al. (40). Micrographs were recorded using a Tecnai 20 electron microscope (FEI, The Netherlands).

Cloning of PHA synthase genes from *H. mediterranei*. To screen PHA synthase genes in *H. mediterranei*, two PCR primers (P1 and P2) (Table 2) were designed based on highly conserved regions of known haloarchaeal PHA synthases (84-L IVYALIN-93 and 317-DHLIPPE-325; the numbering corresponds to *H. marismortui* PhaC ($PhaC_{Hm}$), GenBank accession no. AY596297). The resulting PCR fragment was ligated into the pGEM-T vector (Promega) and sequenced. To clone the full-length PHA synthase genes and the adjacent regions, a thermal asymmetric interlaced (TAIL) PCR (25) was performed. For this, three arbitrary degenerate (AD1 to -3) primers and three interlaced specific forward (dp1 to -3) and reverse (up1 to -3) primers complementary to the known partial $phaC_{Hme}$ nucleotide sequence were designed (Table 2). The TAIL-PCR was performed according to the protocol developed by Liu et al. (24). The secondary and tertiary PCR products were separated by electrophoresis on a 1.0% agarose gel, and the different sizes of the two PCR products consistent with primer positions were used as the criteria for selection of correct TAIL-PCR products. The correct tertiary PCR product was purified and cloned into the pGEM-T vector, and its nucleotide sequence was determined by sequencing.

DNA and protein sequence analysis and phylogenetic tree construction. DNA and deduced amino acid sequences were analyzed with DNASTAR software (2). Sequence homology analysis was performed using the BLAST service (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi) and the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/index.html). The phylogenetic trees for PhaC and PhaE/R were constructed using the neighbor-joining method (34) with MEGA4 (39). The topology of the phylogenetic tree was evaluated by bootstrap analysis on the basis of 1,000 replications (7).

Western blot analysis. For Western blot analysis, crude extracts from *H. mediterranei* cells were obtained by disrupting the cells with ultrasonication as described above. The concentrations of cellular proteins and PHA granule proteins were determined with a bicinchoninic acid protein assay kit (Pierce). One hundred micrograms of proteins from cellular extracts or PHA granules was then subjected to Western analysis with the anti-$PhaE_{Hm}$-His_6 or anti-$PhaC_{Hm}$-His_6 antiserum as described previously (11).

Disruption of the $phaEC_{Hme}$ genes in *H. mediterranei*. The strategy for $phaEC_{Hme}$ gene disruption in *H. mediterranei* CGMCC (China General Microbiological Culture Collection Center) 1.2087 was based on a two-step procedure as described by Tu et al. (41). Briefly, a 677-bp DNA fragment located immediately upstream of the $phaEC_{Hme}$ operon and a 641-bp fragment in the 3′ region of the $phaC_{Hme}$ gene were amplified by primer pairs KF1/KR1 and KF2/KR2 (Table 2), respectively. These two PCR products were sequenced and inserted into the plasmid pUBP (Table 1). The resulting plasmid, pLHC (Table 1), was then transformed into *H. mediterranei* to disrupt the $phaEC_{Hme}$ genes by homologous recombination, generating a $phaEC_{Hme}$-deleted strain called Δ$phaEC_{Hme}$.

Complementation analysis of the $phaEC_{Hme}$ functions in *H. mediterranei* Δ$phaEC_{Hme}$ and *H. hispanica* PHB-1. To determine the functions of the $phaEC_{Hme}$ genes in the PHA-negative mutants, the complementation plasmids of the $phaE_{Hme}$ and/or $phaC_{Hme}$ gene were constructed as follows. Plasmids pWL3E and pWL3EC were constructed by, respectively, cloning of $phaE_{Hme}$ (amplified with primers phaEF1 and phaER1) and $phaEC_{Hme}$ (amplified with primers phaEF1 and phaCR1) into pWL102, with the native promoter of $phaEC_{Hme}$. For the $phaC_{Hme}$ gene alone, the promoter sequence of the $phaEC_{Hme}$ genes (amplified with primers phaEF1 and PER) and the coding sequence of $phaC_{Hme}$ (amplified with primers phaCF1 and phaCR2) were joined and inserted into pWL102, resulting in pWL3C. To investigate the function of the C terminus of $PhaC_{Hme}$, the DNA fragment of $phaEC_{Hme}$ with the truncated 3′ region of $phaC_{Hme}$ was amplified with phaEF1 and phaCRS1 primers and inserted into pWL102, resulting in the plasmid pWL3ECS-1 (Table 1). Each construct was confirmed by sequencing and transformed into *H. mediterranei* Δ$phaEC_{Hme}$ or *H. hispanica* PHB-1 (11) to check if the capability for PHA accumulation had been restored.

Overexpression and purification of $PhaE_{Hme}$ and $PhaC_{Hme}$. For expression of $PhaE_{Hme}$ and $PhaC_{Hme}$ in *H. hispanica* PHB-1, the coding sequences of $phaE_{Hme}$ and $phaC_{Hme}$ were amplified with primer pairs phaEFH/phaERH and phaCFH/phaCRH (Table 2), respectively. With primers phaERH and phaCRH, six histidine codons (His_6) were added to the 3′ end of $phaE_{Hme}$ and $phaC_{Hme}$, respectively. These two PCR products were digested by NdeI and NcoI and cloned into the plasmid pWL102 under the strong promoter of a haloarchaeal heat shock gene (*hsp5*; GenBank accession no. AE004438), resulting in the plasmids pWLEhis and pWLChis (Table 1). The constructs were sequenced and then transformed into *H. hispanica* PHB-1. For isolation of $PhaE_{Hme}$-His_6 and $PhaC_{Hme}$-His_6 proteins, the PHB-1 transformants were cultivated at 37°C in 1-liter flasks in 500 ml AS-168 medium to the stationary growth phase and then were collected by centrifugation. The protein purification steps were performed according to the method described by Plößer and Pfeifer (29), except that phenylmethylsulfonyl fluoride (PMSF) was not added to the lysis buffer.

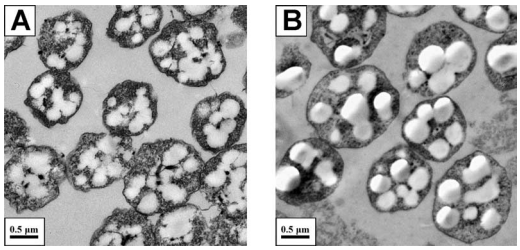

FIG. 1. Electron micrographs of ultrathin sections of *H. mediterranei* demonstrating the accumulation of PHBV granules. The cells were cultured at 37°C for 48 h in AS-168 medium (A) and MST medium (B). The scale bars represent 0.5 μm.

PHA synthase activity assay. PHA synthase activity was measured spectrophotometrically by recording the release of CoA during the polymerization of 3-hydroxybutyryl-CoA. All assays were carried out at 37°C in a final volume of 1 ml containing 20 mM Tris-HCl (pH 7.5), 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 3.4 M KCl, 100 μM $Mg(CH_3COO)_2$, 100 μM 3-hydroxybutyryl-CoA, 1 g/liter bovine serum albumin, and 10 μg of the mixed $PhaE_{Hme}$-His_6 and $PhaC_{Hme}$-His_6 (1:1) proteins. The mixture of $PhaE_{Hme}$-His_6 and $PhaC_{Hme}$-His_6 was stored at 4°C for about 3 h before the activity assay was performed. The absorbance at 412 nm was measured at defined time points. The concentration of CoA was determined using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} (6). One unit was defined as the amount of enzyme that catalyzed the generation of 1 μmol CoA per min.

Nucleotide sequence accession number. The DNA sequences of the $phaEC_{Hme}$ genes of *H. mediterranei* CGMCC 1.2087 reported in this study were deposited in GenBank under accession number EU374220.

RESULTS

PHA accumulation in *H. mediterranei* CGMCC 1.2087. The capability of *H. mediterranei* CGMCC 1.2087 for PHA synthesis in shake flask cultures was investigated in both nutrient-rich AS-168 and nutrient-limited MST media. Interestingly, the strain was capable of synthesizing considerable PHA when cultivated under both growth conditions (Fig. 1). The typical PHA granules accounted for a significant fraction of the cell volume, with shapes and sizes (0.1 to 0.5 μm in diameter) similar to those in bacteria. These PHA granules were further identified as PHBV, as revealed by GC analysis (Table 3). *H. mediterranei* grown in AS-168 and MST media in shaking flasks accumulated PHBV up to 18.21% and 24.85% of the cell dry weight, containing 9.33 mol% and 13.37 mol% 3HV fraction, respectively (Table 3). These results were different from those of most PHA-accumulating microorganisms, which can synthesize detectable PHAs only under nutrient-limiting conditions with excess carbon source (11, 13, 38).

Cloning and identification of PHA synthesis genes in *H. mediterranei*. To clone the PHA biosynthesis genes in *H. mediterranei*, we first amplified a DNA fragment (~700 bp) with two primers (P1 and P2) (Table 2) designed according to the highly conserved regions of known PhaC subunits of haloarchaea. The deduced amino acid sequence encoded by this PCR-amplified DNA showed high homology to PhaC subunits of haloarchaea and some bacteria, indicating that a partial sequence

TABLE 3. PHA accumulation in *H. mediterranei* and *H. hispanica* strains[a]

Strain	Medium	Cell dry wt (g/liter)	PHBV content (% [wt/wt])	3HV fraction (mol%)	PHBV concn (g/liter)
H. mediterranei	AS-168	7.33 ± 0.51	18.21 ± 1.88	9.33 ± 0.13	1.33 ± 0.05
H. mediterranei	MST	7.01 ± 0.35	24.88 ± 1.27	13.37 ± 0.73	1.74 ± 0.04
Δ*phaEC*$_{Hme}$(pWL3E)	MST	9.03 ± 0.14	ND	ND	ND
Δ*phaEC*$_{Hme}$(pWL3C)	MST	9.27 ± 0.15	ND	ND	ND
Δ*phaEC*$_{Hme}$(pWL3EC)	MST	9.02 ± 0.15	14.94 ± 0.24	11.85 ± 0.16	1.35 ± 0.04
Δ*phaEC*$_{Hme}$(pWLEC)	MST	8.43 ± 0.31	17.26 ± 0.08	11.78 ± 0.44	1.38 ± 0.17
H. hispanica	MG	4.70 ± 0.23	12.26 ± 0.52	3.18 ± 0.35	0.58 ± 0.03
PHB-1(pWL3E)	MG	4.14 ± 0.10	ND	ND	ND
PHB-1(pWL3C)	MG	4.20 ± 0.21	ND	ND	ND
PHB-1(pWL3EC)	MG	5.27 ± 0.61	17.33 ± 0.04	3.14 ± 0.13	0.99 ± 0.11
PHB-1(pWL3ECS-1)	MG	6.39 ± 0.43	0.41 ± 0.02	ND	0.03 ± 0.00

[a] The cells were cultured at 37°C for 72 h. The data are shown as mean ± standard deviation; $n = 3$. ND, not detectable.

of the *H. mediterranei* PHA synthase gene (*phaC*$_{Hme}$) was obtained. In order to acquire the entire *phaC*$_{Hme}$ gene and its adjacent genes, a TAIL-PCR was performed to clone the upstream (~1,100-bp) and downstream (~1,700-bp) sequences (Fig. 2A), and a 3,459-bp DNA fragment was cloned and sequenced (Fig. 2B). This DNA region consisted of four open reading frames (ORFs), ORF1 to -4.

ORF2 and ORF3 encode proteins sharing 60% and 57% identity with PhaE and PhaC of *Haloarcula* (11); hence, they were designated *phaE*$_{Hme}$ and *phaC*$_{Hme}$, respectively. The initiation codon (ATG) of *phaE*$_{Hme}$ overlapped the termination condon (TGA) of *phaC*$_{Hme}$, sharing the two bases T and G, and the *phaEC*$_{Hme}$ genes were under the control of a single promoter upstream of *phaE*$_{Hme}$ (see Fig. S1 in the supplemental material). Therefore, these two genes most likely constitute an operon, as observed in *Haloarcula* (11). PhaC$_{Hme}$ (54,765 Da) contained a highly conserved "lipase box-like" sequence (Gly-X-Cys-X-Gly-Gly), which was believed to be an active site of PHA synthase (37). Moreover, the conserved class III PHA synthase box (10) and putative catalytic residues were also found in PhaC$_{Hme}$ (Fig. 3). Interestingly, PhaC$_{Hme}$ and other known haloarchaeal PhaC subunits had longer C termini than the bacterial class III PhaC subunits (Fig. 3), while the haloarchaeal PhaEs are much smaller than their counterparts in bacteria (11), suggesting that these haloarchaeal PHA synthases might constitute a new subgroup of class III PHA synthase.

Upstream and downstream of the *phaEC*$_{Hme}$ genes there are ORF1, encoding an unknown protein, and ORF4, oriented

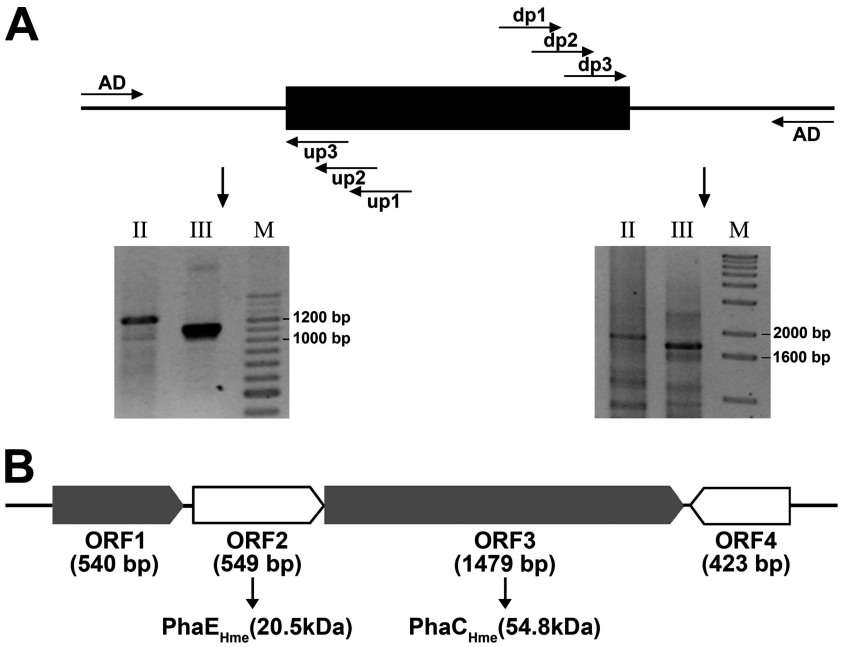

FIG. 2. Cloning and organization of the PHA synthase genes in *H. mediterranei*. (A) Cloning of the PHA synthase genes by TAIL-PCR. The left and right gels represent the second- and third-round (II and III) PCR products, upstream and downstream of the known partial *phaC* sequence, respectively. The interlaced specific primers (dp1 to -3 and up1 to -3) (Table 2) were designed based on the obtained partial *phaC* sequence (black box). AD, arbitrary degenerate primers (AD1 to -3) (Table 2). (B) Gene organization of the cloned DNA region. ORF1 encodes an unknown protein; ORF2 and ORF3 encode PhaE$_{Hme}$ and PhaC$_{Hme}$, respectively; ORF4 encodes a universal stress protein (COG0589).

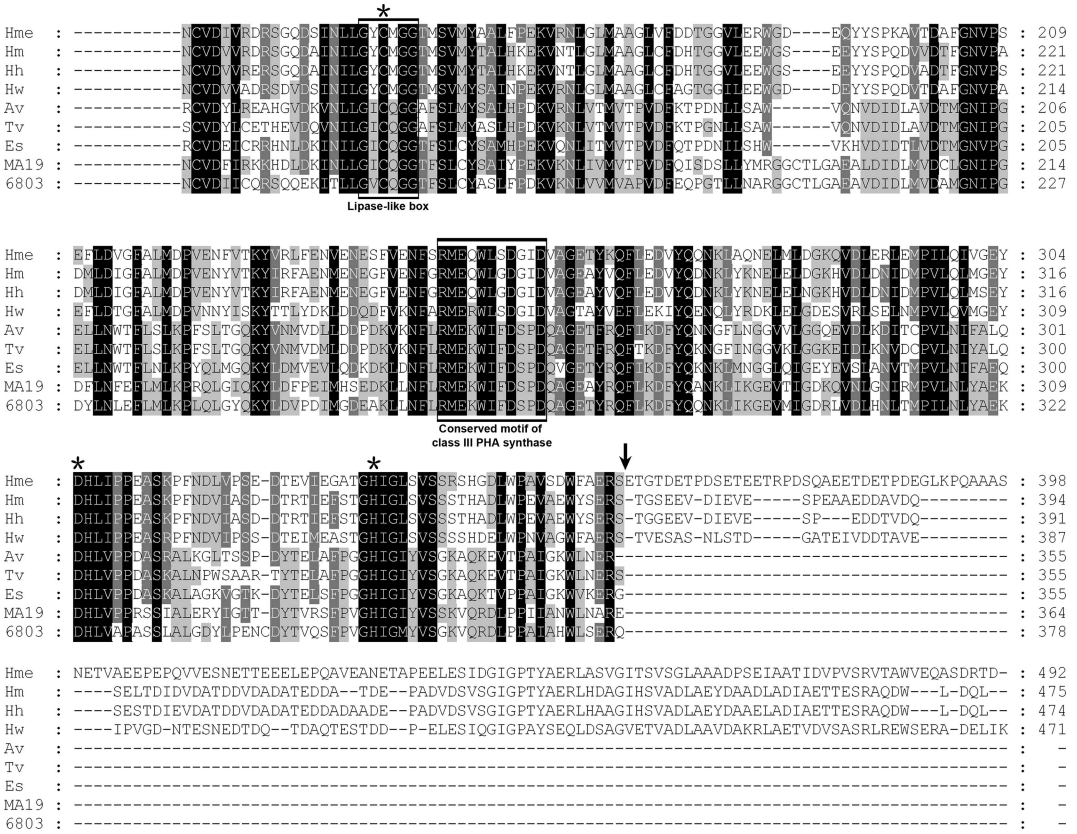

FIG. 3. Multiple alignments of partial amino acid sequences of PhaC subunits from *H. mediterranei* CGMCC 1.2087 (Hme), *H. marismortui* ATCC 43049 (Hm), *H. hispanica* ATCC 33960 (Hh), *H. walsbyi* DSM 16790 (Hw), *Allochromatium vinosum* (Av), *Thiocystis violacea* (Tv), *Ectothiorhodospira shaposhnikovii* (Es), *Synechococcus* sp. strain MA19 (MA19), and *Synechocystis* sp. strain PCC 6803 (6803). Amino acids are given in standard one-letter abbreviations, and the numbers indicate the positions of the amino acids within the respective proteins. The "lipase-like box" and the highly conserved motif of class III synthase are boxed. The conserved catalytic triad residues are shown with asterisks. The vertical arrow indicates the truncated site of the PhaC subunit encoded in pWL3ECS-1. Black shading indicates identical residues, and gray shading indicates similar residues. GenBank accession numbers are as follows: $PhaC_{Hme}$, EU374220; $PhaC_{Hm}$, YP_137339; $PhaC_{Hh}$, ABV71394; $PhaC_{Hw}$, YP_658052; $PhaC_{Av}$, S29274; $PhaC_{Tv}$, AAC60430; $PhaC_{Es}$, AAG30259; $PhaC_{MA19}$, AAK38139; $PhaC_{6803}$, BAA17430.

in the opposite direction to the other three genes, encoding a putative universal stress protein (Fig. 2B). Both ORF1 and ORF4 had their own promoters (data not shown), and their relevance to PHA synthesis remains to be investigated.

Genetic determination of $phaEC_{Hme}$ function in PHBV synthesis. Sequence analysis of the $phaE_{Hme}$ and $phaC_{Hme}$ genes suggested that they might encode the PHA synthase in *H. mediterranei*. To confirm this predication, the $phaEC_{Hme}$ genes in *H. mediterranei* were disrupted with a double-crossover homologous-recombination strategy, which resulted in a $phaEC_{Hme}$-deleted strain called *H. mediterranei* $\Delta phaEC_{Hme}$. The successful deletion of the complete $phaE_{Hme}$ gene, as well as the 5′ region of $phaC_{Hme}$, in *H. mediterranei* $\Delta phaEC_{Hme}$ was proven by PCR analysis. As expected, GC analysis revealed that PHBV accumulation was completely abolished in the $\Delta phaEC_{Hme}$ cells, while the PHA synthase activity of the crude extracts was also totally lost (data not shown). These results confirmed that the $phaEC_{Hme}$ genes are indeed involved in PHBV synthesis in *H. mediterranei*. To further analyze the functions of $PhaE_{Hme}$ and $PhaC_{Hme}$ during PHBV synthesis, the plasmids pWL3E, pWL3C, and pWL3EC were transformed into the $\Delta phaEC_{Hme}$ strain. It was revealed that only the strain harboring pWL3EC exhibited PHA synthase activity in vitro. Consistently, only coexpression of the $phaEC_{Hme}$ genes in *H. mediterranei* $\Delta phaEC_{Hme}$ restored the ability for PHBV accumulation (Table 3). Expression of either $phaE_{Hme}$ or $phaC_{Hme}$ alone in the $\Delta phaEC_{Hme}$ strain could not lead to any detectable PHA synthesis (Table 3). Therefore, our results confirmed that the $phaEC_{Hme}$ genes encoded the PHA synthase in *H. mediterranei*. Intriguingly, when $phaEC_{Hm}$ genes (in pWLEC) from *Haloarcula* (11) were coexpressed in *H. mediterranei* $\Delta phaEC_{Hme}$, the ability for PHBV synthesis in this strain was also fully restored (Table 3), indicating that $PhaEC_{Hme}$ and $PhaEC_{Hm}$ have similar substrate specificities and enzyme activities.

The plasmids pWL3E, pWL3C, pWL3EC, and pWL3ECS-1, harboring a truncated $phaC_{Hme}$ gene (Fig. 3), were also transformed into a PHA-negative archaeon, *H. hispanica* PHB-1 (11). As shown in Table 3, only coexpression of the $phaEC_{Hme}$ genes in *H. hispanica* PHB-1 could restore to this mutant the ability to accumulate PHA to the same level as the wild-type strain. Moreover, *H. hispanica* PHB-1 harboring pWL3ECS-1 accumulated much less PHA than the transformants harboring pWL3EC (Table 3). These results demonstrated that the

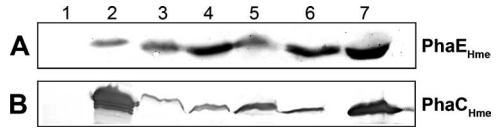

FIG. 4. Western blot analysis of cellular extracts and PHA granules extracted from *H. mediterranei* with antisera against PhaE$_{Hm}$ (A) and PhaC$_{Hm}$ (B). Lanes 1, crude extracts of Δ*phaEC*$_{Hme}$; lanes 2, crude extracts of Δ*phaEC*$_{Hme}$ harboring pWL3EC; lanes 3 and 4, crude extracts of the *H. mediterranei* wild-type strain grown in AS-168 medium for 48 and 72 h, respectively; lanes 5 and 6, crude extracts of the *H. mediterranei* wild-type strain grown in MST medium for 48 and 72 h, respectively; lanes 7, proteins from PHBV granules of *H. mediterranei*. Equal amounts (100 μg) of proteins were loaded on each lane.

phaEC$_{Hme}$ genes encoded a PHA synthase that also functioned in *Haloarcula*, and the longer C-terminal sequence of PhaC$_{Hme}$ was indispensable for this functional PHA synthase. The PHA synthesized in *H. hispanica* has been previously recognized as PHB (11) due to the much lower 3HV content. Interestingly, although transformation of *H. hispanica* PHB-1 with the *phaEC*$_{Hme}$ genes restored the ability to produce PHA, the 3HV content was not elevated (Table 3), suggesting that the different 3HV contents in PHAs of *H. hispanica* and *H. mediterranei* were probably due to the precursor (3HV-CoA) synthesized and not the substrate specificities of the PHA synthases.

Expression and location of PhaC$_{Hme}$ and PhaE$_{Hme}$ in *H. mediterranei*. To further demonstrate the functions of PhaC$_{Hme}$ and PhaE$_{Hme}$ in PHBV synthesis in *H. mediterranei*, Western blotting was performed to analyze the expression profiles of the two proteins and their association with the PHBV granules. Both PhaE$_{Hme}$ and PhaC$_{Hme}$ were detected in cellular extracts and PHBV granules, in either AS-168 or MST medium, in *H. mediterranei*, but not in the *phaEC*$_{Hme}$-deleted strain Δ*phaEC*$_{Hme}$ (Fig. 4). This result suggested that both PhaE$_{Hme}$ and PhaC$_{Hme}$ were constitutively expressed and were stably attached to the PHA granules during PHA synthesis. The protein levels of PhaE$_{Hme}$ and PhaC$_{Hme}$ were influenced little by growth conditions, which might explain the PHBV accumulation of *H. mediterranei* in both nutrient-limited and -rich media (Fig. 1 and Table 3).

***H. mediterranei* PHA synthase is composed of PhaC and PhaE subunits.** To determine that *H. mediterranei* PHA synthase was indeed a two-subunit enzyme, both PhaC$_{Hme}$ and PhaE$_{Hme}$ proteins with His$_6$ tags, expressed under the control of a strong haloarchaeal promoter, were purified from the respective PHB-1 transformants and were subjected to PHA synthase activity assays. Significantly, neither PhaC$_{Hme}$ nor PhaE$_{Hme}$ alone could lead to a detectable PHA synthase activity in vitro, but a 1:1 mixture of the two proteins showed significant activity (~50 U/mg).

Taken together, our genetic and biochemical evidence presented here established that the PHA synthase from *H. mediterranei* was composed of two subunits, PhaE$_{Hme}$ and PhaC$_{Hme}$, and was responsible for PHBV biosynthesis in *H. mediterranei*.

DISCUSSION

PHA accumulation usually occurs under conditions with a limitation of an essential nutrient but in the presence of excess carbon resources (27). However, it has been reported that *Cupriavidus necator* (formerly *Ralstonia eutropha*) can accumulate 2.6% (wt/wt) PHB in a nutrient-rich medium (43), and the PHB content was as high as 35% (wt/wt) transiently in the early growth phase and then decreased gradually (33). In the present study, *H. mediterranei* was revealed to synthesize up to 18% (wt/wt) PHBV at 72 h in nutrient-rich AS-168 medium (Fig. 1A and Table 3), which is different from many bacteria, whose PHA contents were relatively low in nutrient-rich media, and was also distinct from *H. marismortui*, which could accumulate only trace amounts of PHB when cultured in AS-168 medium (11). Consistently, it has been reported that limitation of the nitrogen source has little stimulating effect on PHA production in *H. mediterranei* (23). This is likely because *H. mediterranei* could also synthesize PHBV from amino acids, but the detailed pathway of PHA biosynthesis in *H. mediterranei* remains to be investigated.

Recently, the class III PHA synthase from *Haloarcula* was genetically identified for the first time in the domain *Archaea* in our laboratory (11). However, direct biochemical proofs supporting the notion that this synthase consists of only two subunits are still lacking. In our current study, investigation of the enzyme activities of purified PhaE$_{Hme}$ and PhaC$_{Hme}$ directly proved that the *H. mediterranei* PHBV synthase was indeed composed of two subunits, PhaE and PhaC. Further analysis of the complete genome sequences of *Haloquadratum walsbyi* also suggests that it harbors a locus of PHA synthase genes; one gene is annotated as *phaC*, and the other one in the same operon encodes a homologue of the PhaE subunit. Therefore, the class III PHA synthase is likely widespread in haloarchaea. Phylogenetic trees of PhaC and PhaE/R subunits from some representative bacteria and haloarchaea further suggested that the PHA synthase from haloarchaea belongs to a novel subgroup of the class III family (Fig. 5). Due to the limitation of our knowledge about haloarchaeal PHA biosynthesis, it is still unclear whether other types of PHA synthases also exist in haloarchaea. Interestingly, PHA biosynthesis genes are often clustered in PHA-accumulating bacteria (32), whereas all the known haloarchaeal PHA synthase genes are not clustered with other PHA biosynthesis genes (e.g., *phaAB*), except that *H. walsbyi* harbors a *phaB* gene upstream of the *phaEC* genes. It has been proposed that the PHA biosynthesis genes in haloarchaea might have been acquired from bacteria through horizontal gene transfer (15), and the genes involved in supplying PHA monomer may have been separated from the PHA synthase genes when gene transposition occurred.

While the PhaC subunit probably represents the catalytic subunit of PHA synthase, the detailed function of PhaE remains unclear. The *H. mediterranei* PhaC$_{Hme}$ subunit contains the catalytic domain and conserved residues but has a longer C terminus than its bacterial counterparts (Fig. 3). Deletion of this region in PhaC$_{Hme}$ sharply reduces PHA synthesis ability (Table 3), suggesting the important function of the C-terminal part of this haloarchaeal-type PhaC. Interestingly, all of the known haloarchaeal PhaE subunits (~20 kDa) are much smaller than their bacterial counterparts (~40 kDa). The PhaE$_{Hme}$ subunit has been revealed to be bound to PHA granules like the bacterial PhaE subunits (Fig. 4), but it lacks the conserved domains of bacterial PhaEs, e.g., the PhaE box (9). While the PhaE subunits in

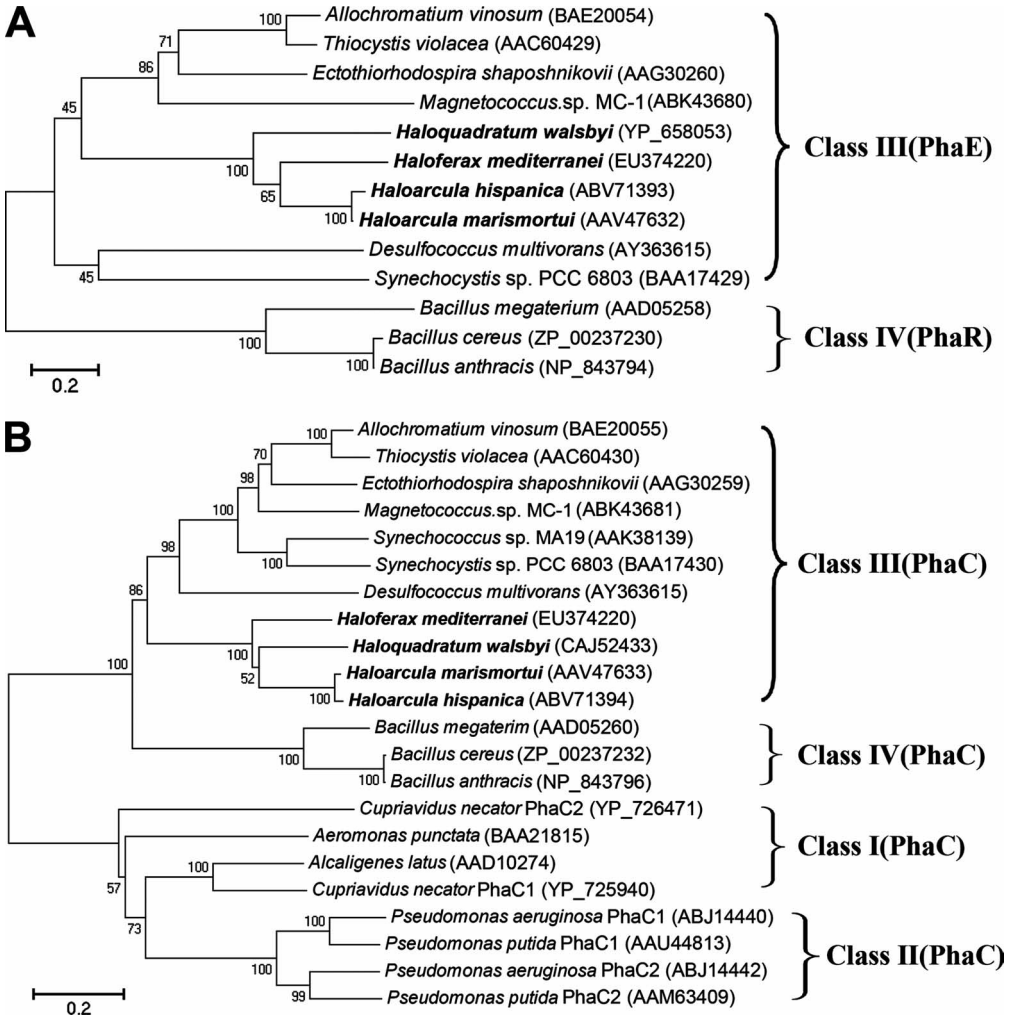

FIG. 5. Phylogenetic trees of four classes of PHA synthases from prokaryotes, including bacteria and haloarchaea (boldface). (A) PhaE or PhaR subunits. (B) PhaC subunits. The phylogenetic trees were constructed based on the amino acid sequence of each protein; the GenBank accession number is given after the microorganism name. The trees were obtained using the neighbor-joining algorithm with MEGA software version 4.0. The numbers next to the nodes indicate the bootstrap values based on 1,000 replications (expressed as percentages). Scale bar = 0.2 substitution per site.

Haloarcula harbor a PhaE box (11), the putative PhaE in *H. walsbyi* also lacks this motif (data not shown). These results further suggest that haloarchaeal PHA synthases constitute a novel subgroup of the class III PHA synthase, and it is imperative to clarify the actual functions of the haloarchaeal-type PhaE subunits in the future.

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