

Wide Distribution among Halophilic Archaea of a Novel Polyhydroxyalkanoate Synthase Subtype with Homology to Bacterial Type III Synthases^{∇†}

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Polyhydroxyalkanoates (PHAs) are accumulated as intracellular carbon and energy storage polymers by various bacteria and a few haloarchaea. In this study, 28 strains belonging to 15 genera in the family Halobacteriaceae were investigated with respect to their ability to synthesize PHAs and the types of their PHA synthases. Fermentation results showed that 18 strains from 12 genera could synthesize polyhydroxybutyrate (PHB) or poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). For most of these haloarchaea, selected regions of the *phaE* and *phaC* genes encoding PHA synthases (type III) were cloned via PCR with consensus-degenerate hybrid oligonucleotide primers (CODEHOPs) and were sequenced. The PHA synthases were also examined by Western blotting using haloarchaeal *Haloarcula marismortui* PhaC (PhaC_{Hm}) antisera. Phylogenetic analysis showed that the type III PHA synthases from species of the Halobacteriaceae and the Bacteria domain clustered separately. Comparison of their amino acid sequences revealed that haloarchaeal PHA synthases differed greatly in both molecular weight and certain conserved motifs. The longer C terminus of haloarchaeal PhaC was found to be indispensable for its enzymatic activity, and two additional amino acid residues (C143 and C190) of PhaC_{Hm} were proved to be important for its *in vivo* function. Thus, we conclude that a novel subtype (IIIA) of type III PHA synthase with unique features that distinguish it from the bacterial subtype (IIIB) is widely distributed in haloarchaea and appears to be involved in PHA biosynthesis.

Haloarchaea are a distinct evolutionary branch of the domain *Archaea*, and they usually comprise the majority of the prokaryotic population in hypersaline environments (31). Most haloarchaea are able to utilize glucose as a carbon source. However, *Halobacterium* (15) and some *Natrialba* (42) and *Natronomonas* (6, 7) strains cannot. In the presence of excess carbon substrates, certain haloarchaea synthesize polyhydroxyalkanoates (PHAs) and deposit them as intracellular granules (27), which has been proposed as an optional standard for describing new haloarchaeal species (32). Compared with members of the domain *Bacteria*, haloarchaea have several advantages as PHA producers; e.g., they utilize unrelated cheap carbon sources, strict sterilization is not needed, and isolation of PHAs from haloarchaea is much easier (11, 13, 27, 34). Thus, haloarchaea have regained attention in biotechnology lately, especially as an alternative source for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) production (11, 20, 28). Although the family *Halobacteriaceae* includes 30 genera, currently, only a few haloarchaeal strains belonging to the genera *Haloferax*, *Haloarcula*, *Haloquadratum*, *Haloterrigena*, *Halorhabdus*, *Halobiforma*, and *Halopiger* are found to accu-

mulate short-chain-length PHAs (scl-PHAs), such as polyhydroxybutyrate (PHB) and PHBV (2, 11–14, 22, 27, 36, 41).

In the pathway of PHA biosynthesis, PHA synthases play a key role by catalyzing the polymerization of (*R*)-3-hydroxyalkanoyl coenzyme A into PHAs. In bacteria, four types of PHA synthases have been distinguished according to their primary structures, substrate specificities, and subunit compositions (35). Types I and II are composed of only one subunit, whereas types III and IV consist of two subunits. The PHA synthases from *Haloarcula marismortui*, *Haloarcula hispanica*, and *Haloferax mediterranei* have been identified and characterized genetically and biochemically, and all need a high salt concentration for enzyme activity (11, 28). The known haloarchaeal PHA synthases are composed of two subunits (PhaE and PhaC) and share the closest identities with bacterial type III PHA synthases (28, 34). However, these haloarchaeal PHA synthases are still quite different from their bacterial counterparts in molecular weight and amino acid sequence, especially the level of identity of PhaEs between haloarchaea and bacteria, which is quite low (~20%) (11, 28). Whether these differences are universal for haloarchaeal strains remains to be answered. Moreover, it is still unknown whether other haloarchaeal strains are able to synthesize PHAs and whether there are other types of PHA synthases. Thus, it is necessary to perform large-scale screening for the PHA synthases in haloarchaea.

In this work, the PHA accumulation ability of 28 haloarchaeal strains from 15 genera were assessed, and the types of their PHA synthases were also characterized. We propose that

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pUCM-T	2.8-kb vector for cloning, Amp ^r	Sangon
pWL102	10.5-kb shuttle vector, Amp ^r Mev ^r	21
pWLEC	12.6 kb, <i>phaEC</i> _{Hm} and its native promoter	11
pWLECS	12.3 kb, 3'-truncated <i>phaEC</i> _{Hm} and its native promoter	This study
pWL-EC1m	12.6 kb, pWLEC-derived plasmid for PhaEC1m (C143A)	This study
pWL-EC2m	12.6 kb, pWLEC-derived plasmid for PhaEC2m (C143S)	This study
pWL-EC3m	12.6 kb, pWLEC-derived plasmid for PhaEC3m (C162A)	This study
pWL-EC4m	12.6 kb, pWLEC-derived plasmid for PhaEC4m (C162S)	This study
pWL-EC5m	12.6 kb, pWLEC-derived plasmid for PhaEC5m (C190A)	This study
pWL-EC6m	12.6 kb, pWLEC-derived plasmid for PhaEC6m (D317A)	This study
pWL-EC7m	12.6 kb, pWLEC-derived plasmid for PhaEC7m (D317N)	This study
pWL-EC8m	12.6 kb, pWLEC-derived plasmid for PhaEC8m (H318Q)	This study
pWL-EC9m	12.6 kb, pWLEC-derived plasmid for PhaEC9m (H346Q)	This study
pWL-EC10m	12.6 kb, pWLEC-derived plasmid for PhaEC10m (W366A)	This study

the PHA synthases widespread in haloarchaea be clustered as a novel subtype (IIIA; A for halophilic *Archaea*) of type III PHA synthases, whereas their counterparts from bacteria might be assigned to subtype IIIB (B for *Bacteria*).

MATERIALS AND METHODS

Microorganisms. Most of the haloarchaeal strains were provided by the China General Microbiological Culture Collection Center (CGMCC). *Haloarcula amylytica* 26-3, *Halonubrum xinjiangense* 25-13, *Halostagnicola larsenii* 24-25, and *Halonubrum litoreum* 12-2 were recently isolated from Yuncheng Salt Lake (China) or solar salterns of Tianjin (China), and they were identified on the basis of 16S rRNA gene sequencing (GenBank accession numbers HM748593 to HM748596, respectively). *Hfx. mediterranei* CGMCC 1.2087 was used as the positive control to study PHA accumulation. *Escherichia coli* JM109 was grown in Luria-Bertani (LB) medium at 37°C (39) and was used as a host for cloning experiments. When needed, ampicillin was added to a final concentration of 100 µg/ml. PHA-negative mutant *Har. hispanica* PHB-1 (11) was used to study the function of the C-terminal truncated *Har. marismortui* PhaC (PhaC_{Hm}) subunit and the key amino acids of PhaC_{Hm} (11). Mevinolin was added to a final concentration of 5 µg/ml for *Har. hispanica* PHB-1 transformants. In all of the experiments, when solid medium was employed, 1.2% (wt/vol) agar was added.

Mutagenesis and plasmid construction. The plasmids used in this study are listed in Table 1. The primers used for DNA amplification and construction of *phaEC*_{Hm} mutants are provided in Table S1 in the supplemental material.

To investigate the function of the C terminus of PhaC_{Hm}, a DNA fragment of *phaEC*_{Hm} with a truncated 3' region of *phaC*_{Hm} was amplified with the primer pair phaEF/phaCRS (see Table S1 in the supplemental material). The fragment was digested with BamHI and KpnI and inserted into plasmid pWL102 (21), resulting in pWLECS. To explore the key amino acids of PhaC_{Hm}, site-directed mutagenesis of PhaC_{Hm} was performed as previously described (23). Briefly, the fragment of *phaEC*_{Hm} from pWLEC (11) was first inserted into the commercial cloning vector pUCm-T (Sangon, China), resulting in T-*phaEC*_{Hm}, which was used as the PCR template. The primers summarized in Table S1 in the supplemental material were designed to introduce the desired mutations into T-*phaEC*_{Hm} with KOD-plus DNA polymerase (Toyobo, Japan). The parental template DNA and the newly amplified mutagenesis primer-containing DNA were treated with DpnI and then introduced into *E. coli* JM109. After sequencing of the DNA, the T-*phaEC*_{Hm}-derived plasmids with specific mutations in the corresponding amino acid were obtained. The *phaEC*_{Hm} fragments, containing the C143A, C143S, C162A, C162S, C190A, D317A, D317N, H318Q, H346Q, and W366A mutations, were then digested with BamHI and KpnI and inserted into pWL102 (21), resulting in pWL-EC1m to pWL-EC10m, respectively. The plasmids were first constructed in *E. coli* JM109 and confirmed by sequencing. Then, they were introduced into *Har. hispanica* PHB-1 with polyethylene glycol-mediated transformation (4). In the PHA accumulation assay, recombinants harboring pWL102 and pWLEC (11) were employed as negative and positive controls, respectively.

Cultivation conditions. All of the haloarchaeal strains were first cultivated to late logarithmic phase at 37°C in one of the following three nutrient-rich media: AS-168 medium, AS-169 medium, or AS-55 medium (11, 24) (see Table 2 for

details). For PHA accumulation analysis, a 5% (vol/vol) inoculum was transferred into 100 ml of fermentation medium in shaking flasks and cultivated for 96 h. The pH was manually adjusted to about 7.2 for neutrophilic haloarchaea and 9.7 for alkaliphilic haloarchaea in fermentation medium. All of the strains grown in AS-168 medium were further cultivated in MG medium, in which glucose was used as the carbon source (11). The strains grown in AS-169 medium were cultivated in nutrient-limited minimal medium, named HSM (containing, per liter, NaCl, 250 g; MgSO₄ · 7H₂O, 20 g; KCl, 2.0 g; yeast extract, 1 g; trisodium citrate, 3.0 g; KH₂PO₄, 37.5 mg; FeSO₄ · 7H₂O, 50 mg; and MnCl₂ · 4H₂O, 0.36 mg; pH 7.2), supplemented with 10 g/liter glycerol or 10 g/liter glucose as the carbon source. The strains grown in AS-55 medium were further cultivated in another nutrient-limited minimal medium, named BM (containing, per liter, NaCl, 200 g; MgSO₄ · 7H₂O, 0.1 g; KCl, 2.0 g; trisodium citrate, 3.0 g; Na₂CO₃, 8.0 g; KH₂PO₄, 37.5 mg; FeSO₄ · 7H₂O, 50 mg; MnCl₂ · 4H₂O, 0.36 mg; and yeast extract, 1 g; pH 9.7), supplemented with 10 g/liter glucose, fructose, or acetate as the carbon source (Table 2). The recombinants of *Har. hispanica* were cultured as previously described (11).

Analysis of PHA accumulation. After 96 h of fermentation, haloarchaeal cells were harvested by centrifugation and lyophilized overnight. The cellular PHA content and its composition were analyzed by gas chromatography with a GC-6820 chromatograph (Agilent) after methanolysis (11), and PHBV (Sigma) was used as the standard. The PHA contents (wt%) in the cells were calculated as (mass of PHA/original lyophilized cell mass) × 100%.

Genomic isolation and CODEHOP PCR. All of the total genomic DNA of the halophiles tested in this study was extracted from late-logarithmic cultures according to the method of DasSarma et al. (5). Consensus-degenerate hybrid oligonucleotide primers (CODEHOPS) (37) were designed from highly conserved blocks of multiply aligned protein sequences of eight published haloarchaeal PhaE and PhaC subunits (<http://blocks.fhcr.org/codehop.html>). We obtained two optimal primer pairs, codehopEF/codehopER (5'-CGACCGAGTTCCGCGAYATHTGGYT-3' and 5'-GCGTGCTGGCGGCKYTCNAVYTC-3') for the *phaE* region and codehopCF/codehopCR (5'-ACCGACGTCGTCTAC AAGGARAAYAARYT-3' and 5'-GGTCGCGGACGACGTCNACRCARTT-3') for the *phaC* region.

In the PCR assay, total DNA extracted from the 28 tested haloarchaeal strains was used as the template, and the DNA of *Hfx. mediterranei* CGMCC 1.2087 was used as the positive control. The PCR products (~230 bp for *phaE*, ~280 bp for *phaC*) were run on a 1% agarose gel and purified using a gel extraction kit (Bioflux), following the instructions of the manufacturer. The purified DNA fragments were subsequently inserted into the commercial cloning vector pUCm-T (Sangon, China) in *E. coli* JM109 for further sequencing.

Conserved sequence analysis and phylogenetic tree construction. The cloned fragments were sequenced using the universal M13 forward primer. The deduced amino acid sequences were analyzed with DNASTar software (3). Sequence homology was assessed using the GeneDoc program (<http://www.nrbsc.org/gfx/genedoc/index.html>). The phylogenetic trees for PhaC and PhaE were constructed using the neighbor-joining method (38) with Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.0 (40). The topology of the phylogenetic tree was evaluated by bootstrap analysis on the basis of 1,000 replications (8).

TABLE 2. Summary of three approaches to identifying PHA synthases in haloarchaeal strains used in this study^a

Strain	Nutrient-rich medium/carbon source	PHA accumulation			PCR result		WB result for anti-PhaC _{Hm}
		CDW (g/liter)	PHA content (wt%)	3HV fraction (mol%)	<i>phaE</i>	<i>phaC</i>	
<i>Haloferax mediterranei</i> CGMCC 1.2087	AS-168/glucose	7.0 ± 1.4	16.4 ± 3.3	12.4 ± 0.3	+	+	+
<i>Halalkalicoccus tibetensis</i> CGMCC 1.3240	AS-55/glucose	2.9 ± 0.2	8.1 ± 0.4	2.4 ± 0.1	+	+	+
<i>Haloarcula amylolytica</i> 26-3	AS-168/glucose	2.5 ± 0.4	4.4 ± 0.5	4.2 ± 0.9	+	+	+
<i>Haloarcula argentinensis</i> CGMCC 1.7094	AS-168/glucose	3.3 ± 0.2	6.5 ± 0.7	3.9 ± 0.1	+	+	+
<i>Halobacterium cutirubrum</i> CGMCC 1.1962	AS-169/glycerol	2.4 ± 0.2	7.1 ± 0.3	10.1 ± 1.6	-	-	<+
<i>Halobacterium halobium</i> PM CGMCC 1.1952	AS-169/glycerol	2.6 ± 0.6	5.3 ± 1.4	5.9 ± 0.6	-	-	<+
<i>Halobiforma nitratireducens</i> CGMCC 1.1980	AS-55/fructose	0.38 ± 0.0	5.1 ± 1.5	ND	+	+	+
<i>Halococcus morrhuae</i> CGMCC 1.2153	AS-169/glucose	2.1 ± 0.2	7.0 ± 0.8	9.0 ± 0.6	+	+	+
<i>Haloferax denitrificans</i> CGMCC 1.2198	AS-168/glucose	8.1 ± 1.2	ND	ND	-	-	-
<i>Haloferax gibbonsii</i> CGMCC 1.2148	AS-168/glucose	2.4 ± 0.4	12.7 ± 0.4	2.8 ± 0.5	+	+	+*
<i>Halorubrum litoreum</i> 12-2	AS-168/glucose	2.5 ± 0.1	2.1 ± 0.5	ND	+	+	+*
<i>Halorubrum saccharovorum</i> CGMCC 1.2147	AS-168/glucose	2.2 ± 0.4	ND	ND	-*	+	+*
<i>Halorubrum trapanicum</i> CGMCC 1.2201	AS-168/glucose	1.9 ± 0.1	12.7 ± 3.5	1.2 ± 0.0	+	+	+*
<i>Halorubrum xinjiangense</i> 25-13	AS-168/glucose	3.4 ± 0.2	ND	ND	-	-	+*
<i>Halostagnicola larsenii</i> 24-25	AS-168/glucose	1.7 ± 0.2	1.7 ± 0.2	1.1 ± 0.2	+	+	+*
<i>Haloterrigena turkmenica</i> CGMCC 1.2364	AS-168/glucose	3.0 ± 0.5	4.9 ± 0.6	12.9 ± 0.7	+	+	+*
<i>Natrialba chahannaensis</i> CGMCC 1.1977	AS-55/glucose	1.54 ± 0.2	ND	ND	-*	+	+
<i>Natrialba hulunbeirensis</i> CGMCC 1.1986	AS-55/fructose	0.9 ± 0.0	ND	ND	-	-	+
<i>Natrialba magadii</i> CGMCC 1.1966	AS-55/acetate	0.4 ± 0.0	ND	ND	-	-	+*
<i>Natrialba</i> sp. CGMCC 1.1968	AS-55/glucose	2.2 ± 0.1	ND	ND	+	+	>+
<i>Natrinema altunense</i> CGMCC 1.3731	AS-169/glucose	5.8 ± 0.1	9.1 ± 0.7	16.0 ± 0.2	+	+	+*
<i>Natrinema pallidum</i> JCM 8980	AS-169/glucose	3.5 ± 0.3	22.9 ± 1.4	13.9 ± 1.0	+	+	+*
<i>Natrinema pellirubrum</i> JCM 10476	AS-169/glucose	2.2 ± 0.1	11.5 ± 0.6	ND	+	+	+*
<i>Natrinema</i> sp. XA3-1	AS-169/glucose	1.6 ± 0.2	5.4 ± 0.2	11.4 ± 0.6	+	+	+*
<i>Natronobacterium chagannuoerensis</i> CGMCC 1.1970	AS-55/glucose	1.1 ± 0.1	ND	ND	-	-	+*
<i>Natronobacterium gregoryi</i> CGMCC 1.1967	AS-55/glucose	0.8 ± 0.1	0.8 ± 0.3	ND	+	+	+
<i>Natronococcus occultus</i> CGMCC 1.1964	AS-55/glucose	1.9 ± 0.3	ND	ND	+	+	-
<i>Natronomonas pharaonis</i> CGMCC 1.1965	AS-55/acetate	0.3 ± 0.1	ND	ND	-	-	-
<i>Natronorubrum tibetense</i> CGMCC 1.2123	AS-55/glucose	0.41 ± 0.1	3.6 ± 0.6	2.0 ± 0.7	+	+	+

^a The cells were cultured in nutrient-rich medium to late logarithmic phase at 37°C and then transferred to PHA accumulation medium for 96 h. Data are shown as means ± standard deviations, *n* = 3. ND, not detectable; +, detectable; -, not detectable; -*, the deduced amino acids from the cloned sequences were not the proposed PhaE sequence; >+, the WB band is larger than the band for PhaC_{Hm}; <+, the WB band is smaller than the band for PhaC_{Hm}; +*, multiple cross-reacting bands are detected.

WB analysis. Haloarchaeal strains cultivated under fermentation conditions for 72 h were harvested by centrifugation, washed twice with buffer A (20 mM Tris-HCl, 3 M KCl [pH 7.5]), and then resuspended in an appropriate volume of buffer A. Cells were disrupted by ultrasonication. Cell debris and undissolved material were removed by centrifugation (10,000 × *g*, 30 min, 4°C). Prior to Western blot (WB) analysis, the samples were desalted according to the methods described by Klein et al. (19) and Karadzic and Maupin-Furlow (18) with minor modifications. Briefly, the supernatant was precipitated with 50% (vol/vol) acetone overnight at 4°C, followed by centrifugation (10 min, 10,000 × *g*, 4°C). The resulting white pellets were washed five times with cold acetone and resuspended in sample application buffer (2% [wt/vol] SDS, 12% [wt/vol] glycerol, 120 mM dithiothreitol, 70 mM Tris HCl, pH 6.8). The concentrations of cellular proteins were determined with a bicinchoninic acid protein assay kit (Pierce). About 100 µg of protein from the tested strains or 5 µg of purified His₆-tagged PhaC_{Hm} from *E. coli* was used for Western blot analysis, which was performed as described previously (11) except for some modifications. After incubation with anti-PhaC_{Hm}-His₆ and appropriate washing, the blots were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG. After the final wash, the blot was immersed in a substrate solution containing luminol for HRP (Vigorous Biotechnology, China). Then, X-ray film contact exposure was used to capture the luminescent signal.

RESULTS AND DISCUSSION

PHA accumulation in haloarchaea. The PHA accumulation capacity of 28 strains belonging to 15 haloarchaeal genera was investigated. Six media were prepared for these strains (see Materials and Methods and Table 2) to facilitate growth and PHA accumulation. Most strains could use glucose as a carbon

source; thus, excess glucose was supplied for PHA synthesis for these tested strains. For the other strains, *Halobacterium cutirubrum* CGMCC 1.1962 and *Halobacterium halobium* CGMCC 1.1952 were supplied with excess glycerol, *Halobiforma nitratireducens* CGMCC 1.1980 and *Natrialba hulunbeirensis* CGMCC 1.1986 were supplied with fructose, and *Natrialba magadii* CGMCC 1.1966 and *Natronomonas pharaonis* CGMCC 1.1965 were supplied with acetate (Table 2). All cultures of neutrophilic haloarchaeal strains reached stationary phase after 96 h of fermentation. In contrast, 4 of 11 species of alkaliphilic haloarchaea exhibited relatively poor growth (cell dry weight [CDW], <0.5 g/liter), including *Hbf. nitratireducens* CGMCC 1.1980, *Nab. magadii* CGMCC 1.1966, *Natronococcus occultus* CGMCC 1.1965, and *Natronorubrum tibetense* CGMCC 1.2123.

The levels of PHA accumulation of these strains are summarized in Table 2, in which *Hfx. mediterranei* CGMCC 1.2087 (28) was used as a positive control. In total, 18 of the 28 tested strains were capable of synthesizing PHB or PHBV at levels ranging from 0.8% to 22.9% (wt/wt) of CDW, while formation of medium-chain-length PHA was not observed. The PHA producers were distributed in 12 genera (Table 2), and 8 of these genera were found for the first time to accumulate scl-PHAs. They are *Halalkalicoccus*, *Halobacterium*, *Halococcus*, *Halorubrum*, *Halostagnicola*, *Natrinema*, *Natronobacterium*,

TABLE 3. Selected PHA synthase genes deposited in the NCBI and EMBL databases

Strain	Abbr. ^a	PhaE			PhaC		
		No. of AA	Molecular mass (kDa)	Accession no.	No. of AA	Molecular mass (kDa)	Accession no.
<i>Halobacteriaceae</i>							
<i>Haloarcula marismortui</i>	Hm	181	20.6	YP_137338	475	53.1	YP_137339
<i>Haloarcula hispanica</i>	Hh	181	20.6	ABV71393	475	53.0	ABV71394
<i>Haloferax mediterranei</i>	Hf	182	20.4	ACB10369	492	54.7	ACB10370
<i>Halomicrobium mukohataei</i>	Hmm	181	20.6	YP_003176826	464	51.7	YP_003176827
<i>Halorhabdus utahensis</i>	Hu	182	20.3	YP_003131062	464	52.0	YP_003131063
<i>Halogeometricum borinquense</i>	Hb	184	20.7	ZP_03999958	446	50.1	ZP_03999959
<i>Haloterrigena turkmenica</i>	Ht	181	20.8	YP_003404007	530	58.8	YP_003404006
<i>Haloquadratum walsbyi</i>	Hw	184	20.8	YP_658053	471	52.1	YP_658052
<i>Bacteria</i>							
<i>Allochromatium vinosum</i>	Av	357	40.5	BAE20054	355	39.8	BAE20055
<i>Ectothiorhodospira shaposhnikovii</i>	Es	371	42.0	AAG30260	355	40.2	AAG30259
<i>Synechocystis</i> sp. strain PCC6803	Ssp	330	38.0	BAA17429	378	43.0	BAA17430
<i>Thiocystis violacea</i>	Tv	364	41.5	AAC60429	355	39.6	AAC60430
<i>Desulfococcus multivorans</i>	Dm	306	41.7	AY363615	371	42.5	AY363615

^a Abbr., abbreviation of the haloarchaeal or bacterial name used in Fig. 2 and in Fig. S1 in the supplemental material.

and *Natronorubrum*. Thus, including the 7 previously reported genera, at least 15 genera of haloarchaea have been found to accumulate scl-PHAs. As in *Har. marismortui* and *Har. hispanica* (11, 28), PHBV with a low molar fraction of 3-hydroxyvalerate (3HV) was detected in the other two tested *Haloarcula* species, *Har. amylolytica* and *Haloarcula argentinensis* (Table 2). Interestingly, the two *Halobacterium* strains were also found to synthesize PHBV from glycerol (Table 2), apparently distinguishing them from the two genome-sequenced *Halobacterium salinarum* strains (NRC-1 and R1), as no PHA synthase genes were ever annotated in their genome sequences (30, 33). Similarly, *Haloferax gibbonsii* (but not *Haloferax denitrificans*) and two of the four tested *Halorubrum* species produced PHB or PHBV (Table 2). Interestingly, none of the three *Natrialba* species produced PHAs, but all four of the *Natrinema* species did, with the levels ranging from 5.4 to 22.9% of CDW. It was noteworthy that in *Natrinema pallidum* JCM 8980, the cellular content of PHBV reached ~23% of CDW without culture condition optimization, indicating that this strain might be another promising PHBV producer (Table 2). In summary, scl-PHAs were widely produced by neutro-

philic haloarchaea and a few alkaliphilic haloarchaea, especially when excess glucose was available.

Screening for haloarchaeal PHA synthase genes via CODEHOP PCR. To identify the possible PHA synthases in our strains, we first searched for and analyzed the published genes encoding PHA synthases in haloarchaea. Up to now, there have been eight PHA synthase sequences of haloarchaea deposited in the NCBI database; these are from the strains *Har. marismortui* ATCC 43049, *Har. hispanica* CGMCC 1.2049, *Hfx. mediterranei* CGMCC 1.2087, *Halomicrobium mukohataei* DSM 12286, *Halorhabdus utahensis* DSM 12940, *Halogeometricum borinquense* DSM 11551, *Haloterrigena turkmenica* DSM 5511, and *Haloquadratum walsbyi* DSM 16790. All eight PHA synthases exhibit high levels of homology with each other (PhaC, 58 to 95%; PhaE, 43 to 96%) (Table 3). According to the highly conserved regions in PhaE and PhaC, two pairs of CODEHOPs, codehopEF/codehopER and codehopCF/codehopCR, were designed for the screening of *phaE* and *phaC*, respectively.

Notably, most of the strains (20 of 28) gave single PCR bands consistent with the predicted sizes of both *phaE* (Fig. 1A) and *phaC* (Fig. 1B). In addition to the positive control, 16

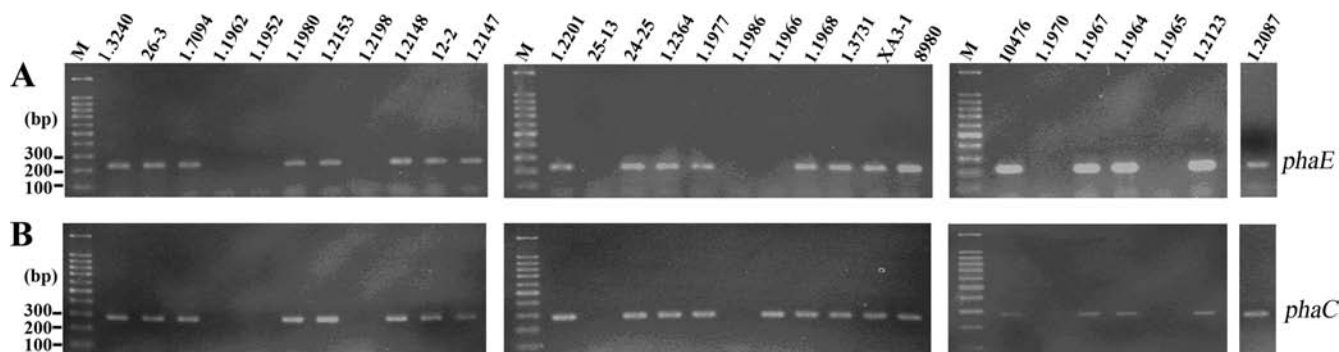


FIG. 1. Cloning of partial sequences of *phaE* (A) and *phaC* (B) via the CODEHOP PCR approach. Lanes M, 100-bp DNA marker. The strain number of the investigated haloarchaeon (Table 2) is indicated. *Hfx. mediterranei* CGMCC 1.2087 was used as a positive control. For primers, see Materials and Methods.

of the 18 PHA-producing strains (the exceptions being *Hbt. halobium* CGMCC 1.1952 and *Hbt. cutirubrum* CGMCC 1.1962) yielded specific amplification products of both *phaE* and *phaC*. Because the two *Halobacterium* strains were able to synthesize PHAs, their genome sequences were presumed to encode a PHA synthase. The absence of PCR products was therefore likely due to the presence of a less homologous PHA synthase in these strains. On the other hand, *phaE* and *phaC* PCR signals were detected for four PHA-negative strains, including *Natrialba chahannaensis* CGMCC 1.1977, *Halo-rubrum saccharovororum* CGMCC 1.2147, *Natrialba* sp. strain CGMCC 1.1968, and *Natronococcus occultus* CGMCC 1.1964. Presumably, these genes were not expressed or the choice of an improper carbon source resulted in no PHA accumulation. For *Nab. magadii* CGMCC 1.1966, another PHA-negative strain, a PCR signal from only *phaC* was obtained (Fig. 1B). The remaining five strains, *Hfx. denitrificans* CGMCC 1.2198, *Hrr. xinjiangense* 25-13, *Nab. hulunbeirensis* CGMCC 1.1986, *Natronobacterium chagannuoerensis* CGMCC 1.1970, and *Nnm. pharaonis* CGMCC 1.1965, gave no PCR products, consistent with the lack of detectable PHAs in these strains (Table 2). In total, the selected regions of the *phaE* and *phaC* genes were amplified from most of the tested strains, indicating that this type of PHA synthase is widespread in haloarchaeal strains.

Screening for PhaC-containing strains using His₆-tagged PhaC_{Hm} antiserum. WB analysis with a haloarchaeal PhaC_{Hm} antiserum (11) was used to confirm the wide distribution of the haloarchaeal type PHA synthase in haloarchaea. The WB results are summarized in Table 2. Up to 25 of the 28 strains exhibited visible reaction signals with the PhaC_{Hm} antiserum. The 3 strains lacking a WB signal were also unable to synthesize detectable PHAs (Table 2).

As observed in the positive control, a specific PhaC signal was detected in the crude extracts of *Halalkalicoccus tibetensis* CGMCC 1.3240, *Har. amylolytica* 26-3, *Har. argentinensis* CGMCC 1.7094, *Hbf. nitratireducens* CGMCC 1.1980, *Halococcus morrhuae* CGMCC 1.2153, *Nrr. tibetense* CGMCC 1.2123, *Natronobacterium gregoryi* CGMCC 1.1967, *Nab. chahannaensis* CGMCC 1.1977, and *Nab. hulunbeirensis* CGMCC 1.1986 (Table 2). In addition, crude extracts from *Natrialba* sp. strain CGMCC 1.1968, *Hbt. halobium* CGMCC 1.1952, and *Hbt. cutirubrum* CGMCC 1.1962 also gave specific bands corresponding to proteins with different molecular weights. The remaining 13 strains gave two or more cross-reacting bands (Table 2). This phenomenon might be a result of the existence of multiple paralogous PhaC proteins, the instability of PhaC during handling and storage, or the presence of a larger complex of PhaC protein with other proteins. In conclusion, the WB analysis provided additional evidence that this haloarchaeal type of PHA synthase is widely distributed in the haloarchaeal strains investigated in this study.

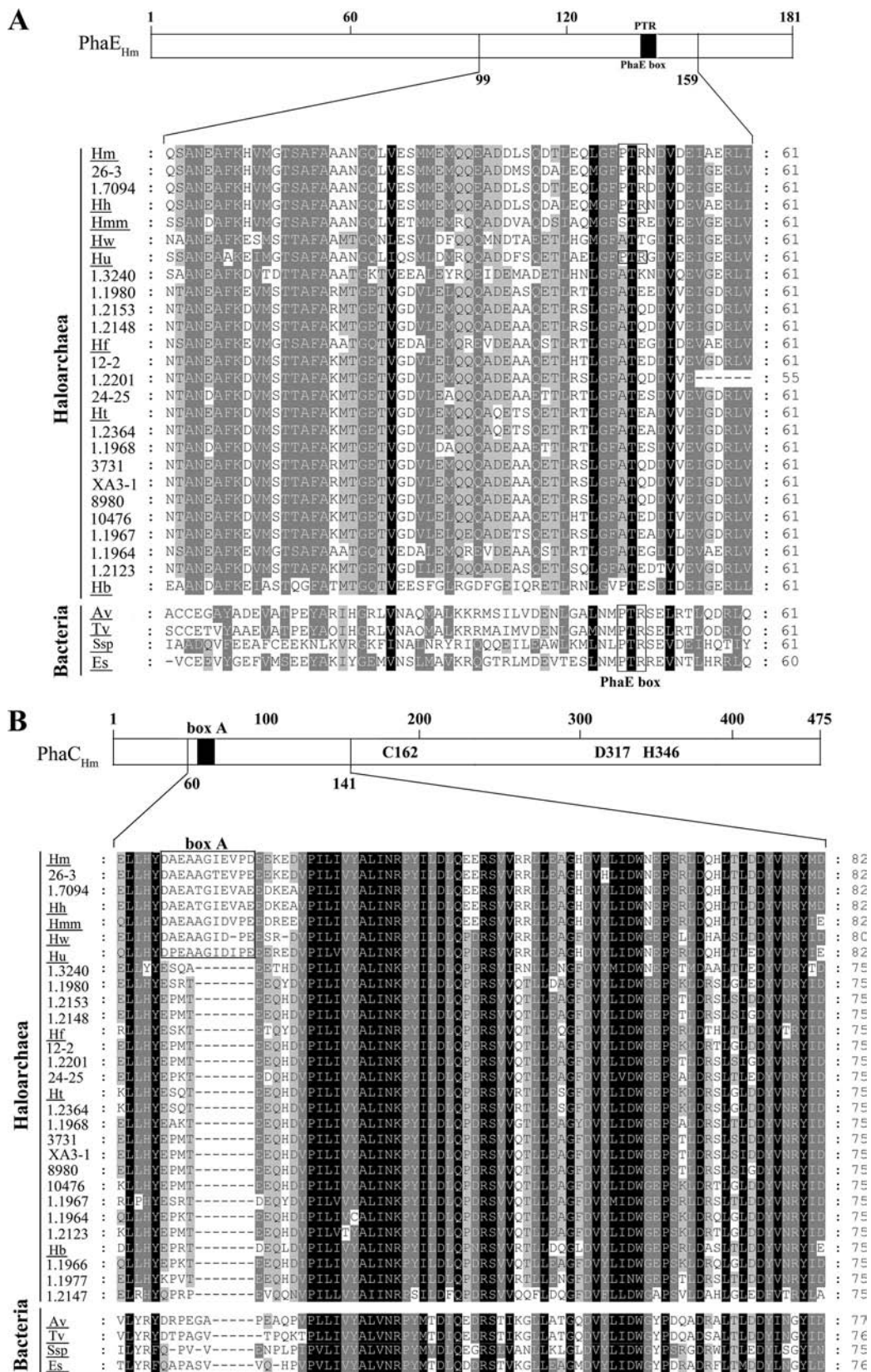
Sequence and phylogenetic analyses of conserved regions of type III PHA synthases. The amplified PCR products were cloned into the vector pUCm-T and sequenced. With the exception of the two sequences (for *phaE*) from *Nab. chahannaensis* CGMCC 1.1977 and *Hrr. saccharovororum* CGMCC 1.2147, the sequences of the conserved PhaE and PhaC regions could be deduced from the cloned fragments (Table 2). To facilitate comparisons among the results of the three different

approaches employed in this study, they are all summarized in Table 2.

The type III PHA synthases from many bacteria and two haloarchaea have been studied biochemically and genetically (10, 11, 25, 26, 28), and in total, 8 haloarchaeal PHA synthases encoding genes are now available in the NCBI database (Table 3). To explore the homology of the type III PHA synthases from the two groups of prokaryotes, the highly conserved sequences obtained in this study and several typical bacterial sequences (Table 3) were aligned. The PhaE (Fig. 2A) and PhaC (Fig. 2B) sequences from the haloarchaeal strains themselves exhibited high levels of similarity, whereas the haloarchaeal PhaEs showed much lower levels of identity with bacterial PhaEs (Fig. 2A). Notably, the PhaE box of Pro-Thr-Arg was highly conserved in all of the bacteria (10), while this box was not so conserved in haloarchaeal PhaEs and was present only in the PhaE sequences of four *Haloarcula* strains and *Hrd. utahensis* DSM 12940 (Fig. 2A). The PhaC sequences from the two groups seemed to be more conserved than the PhaE subunits, displaying several consensus blocks. Interestingly, a region (Fig. 2B, box A) at the N terminus of PhaCs from four *Haloarcula* strains (*Har. marismortui* ATCC 43049, *Har. hispanica* CGMCC 1.2049, *Har. amylolytica* 26-3, and *Har. argentinensis* CGMCC 1.7094) and other haloarchaeal strains (*Hmc. mukohataei* DSM 12286, *Hqa. walsbyi* DSM 16854, and *Hrd. utahensis* DSM 12940) distinct from the N terminus of PhaCs of the rest of the strains was observed (Fig. 2B). The alignment analysis showed that although these PHA synthases from the *Haloarchaea* and *Bacteria* belonged to type III, they still exhibited somewhat different amino acid sequences even within these conserved regions (Fig. 2).

To thoroughly understand the phylogenetic relationships of these homogeneous PHA synthases, two phylogenetic trees were generated on the basis of the selected conserved sequences of PhaE and PhaC from haloarchaea and four bacteria (*Allochrochromatium vinosum*, *Thiocystis violacea*, *Synechocystis* sp. strain PCC 6803, and *Ectothiorhodospira shaposhnikovii*) (Fig. 3). Both PhaEs (Fig. 3A) and PhaCs (Fig. 3B) within the haloarchaeal group were clearly clustered together and were only distantly related to those of the bacterial group, which was consistent with the findings of alignment analysis (Fig. 2B). It has been suggested that horizontal gene transfer might have occurred in type III synthases between bacteria and halophilic archaea but that those in haloarchaea have diverged with their own features (34).

To reveal other unique features of haloarchaeal PHA synthases, all known full-length haloarchaeal PHA synthases (Table 3) were further compared with typical bacterial type III PHA synthases. As observed previously (11, 28), the molecular mass of all haloarchaeal PhaE subunits is about 20 kDa (range, 20.1 to 20.8 kDa), whereas haloarchaeal PhaC subunits display a wider molecular mass distribution, from 50.1 to 58.8 kDa (Table 3). The molecular masses of these two subunits are obviously distinct from those of the corresponding subunits of bacterial type III PHA synthase, which are about 40 kDa (10, 25, 26). Compared with bacterial PhaCs, all of the haloarchaeal PhaC subunits have a longer sequence at their C terminus (see Fig. S1A in the supplemental material), which might endow haloarchaeal PhaCs with high hydrophobicity in this region. In



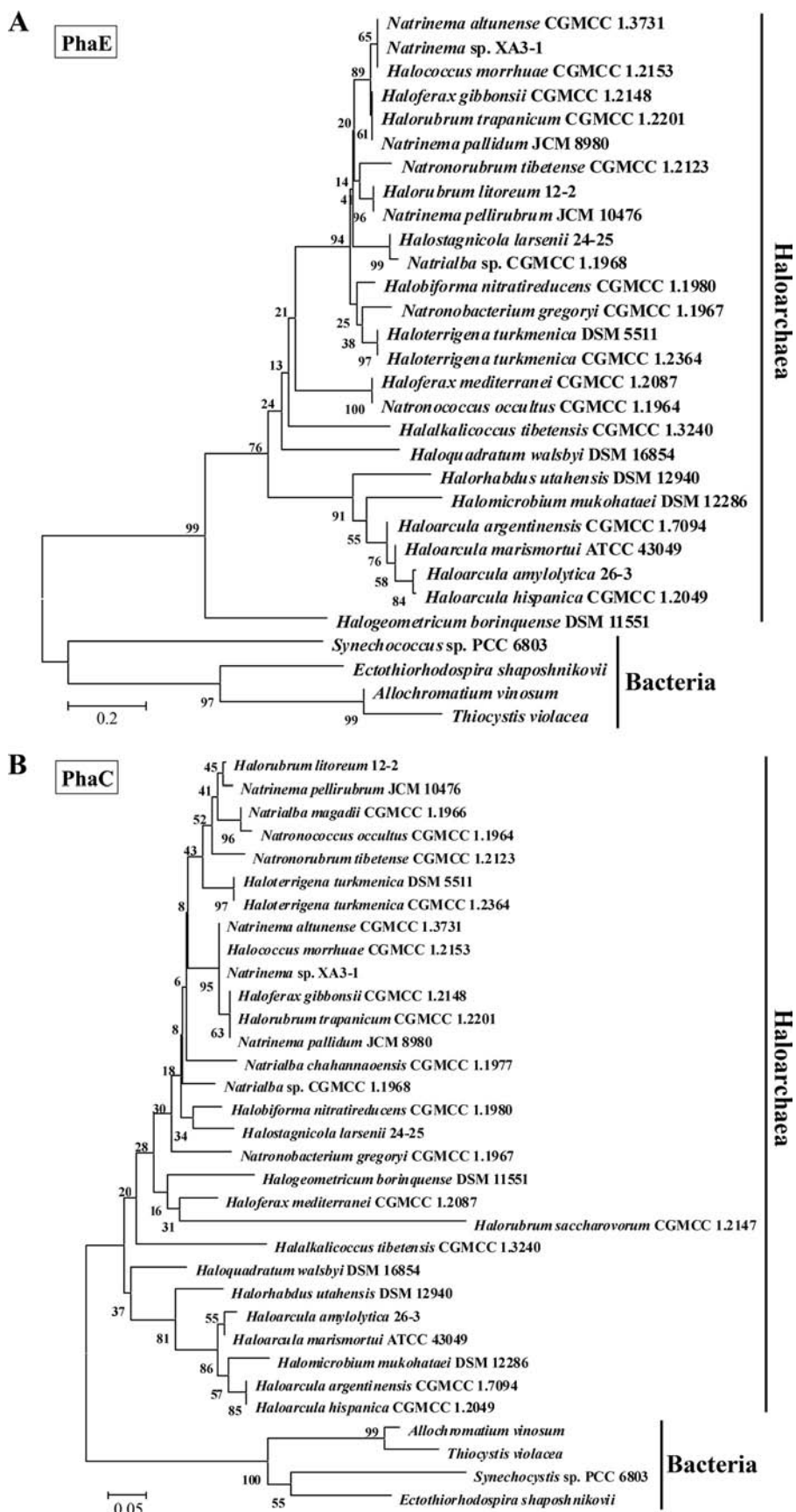


FIG. 3. Phylogenetic analysis of the conserved sequences of PhaE (A) and PhaC (B) from *Halobacteriaceae* and *Bacteria* (same strains as in Fig. 2). The trees were constructed using the neighbor-joining algorithm with MEGA software, version 4.0. The numbers next to the nodes indicate the bootstrap values based on 1,000 replications (expressed as percentages). Scale bars, 0.2 (PhaE) and 0.05 (PhaC) substitutions per site.

TABLE 4. PHA accumulation in *Har. hispanica* PHB-1 recombinant strains^a

Strain	CDW (g/liter)	PHA content (wt%)	Relative PHA concn (%)
PHB-1(pWL102)	4.72 ± 0.51	ND	0
PHB-1(pWLEC)	4.07 ± 0.02	11.9 ± 0.13	100
PHB-1(pWLECS)	4.12 ± 0.37	5.02 ± 0.30	43.8
PHB-1(pWL-EC1m, C143A)	4.65 ± 0.47	1.24 ± 0.22	12.5
PHB-1(pWL-EC2m, C143S)	4.80 ± 0.35	8.0 ± 0.10	80
PHB-1(pWL-EC3m, C162A)	5.38 ± 0.36	0.22 ± 0.02	2.0
PHB-1(pWL-EC4m, C162S)	4.35 ± 0.27	0.69 ± 0.05	8.0
PHB-1(pWL-EC5m, C190A)	5.11 ± 0.13	0.15 ± 0.03	2.1
PHB-1(pWL-EC6m, D317A)	5.11 ± 0.64	0.21 ± 0.01	2.2
PHB-1(pWL-EC7m, D317N)	4.87 ± 0.14	1.43 ± 0.11	14.5
PHB-1(pWL-EC8m, H318Q)	3.01 ± 0.18	7.95 ± 0.41	58.3
PHB-1(pWL-EC9m, H346Q)	2.76 ± 0.14	0.42 ± 0.03	2.0
PHB-1(pWL-EC10m, W366A)	4.51 ± 0.22	5.76 ± 0.85	54.1

^a The cells were first cultured in nutrient-rich medium at 37°C to late logarithmic phase and then transferred to PHA accumulation medium for 96 h. Data are shown as means ± standard deviations, *n* = 3. ND, not detectable.

contrast, bacterial PhaEs have a longer sequence at the N terminus than haloarchaeal PhaEs.

In summary, the type III PHA synthases from the bacteria and haloarchaea have been developed into two subgroups distinctive in the molecular masses and certain conserved motifs of their subunits.

Importance of the longer sequence at C terminus of haloarchaeal PhaCs. The longer C-terminal sequence of PhaC from *Hfx. mediterranei* has been shown to be indispensable for the polymerization activity of PHA synthase (28). We next asked whether this was the case with other haloarchaeal PhaC subunits, and the PHA synthase from the genus *Haloarcula* was used to explore the function of the longer C-terminal sequence of the PhaC subunit (the truncated site is shown in Fig. S1A in the supplemental material). The *in vivo* activity of mutant PHA synthase was measured by the ability to accumulate PHA (relative PHA concentration, in percent), which, for the haloarchaeal strains harboring the wild-type PHA synthase, was arbitrarily defined as 100% (Table 4). Fermentation results revealed that the PHA content declined from 11.90 wt% to 5.02 wt% after the C terminus of PhaC_{Hm} was truncated (Table 4). Correspondingly, the level of PHA accumulation was reduced by more than half (43.8% of that for the wild type) (Table 4). These results indicate that the longer C-terminal sequence of PhaC_{Hm} plays an important role in PHA polymerization. Thus, we conclude that the longer sequence located at the haloarchaeal PhaC C terminus is necessary for full enzymatic activity.

Genetic identification of important residues of PhaC_{Hm}. In order to identify the important amino acids of the haloarchaeal PHA synthases and compare the sequences with those of their bacterial counterparts, mutagenesis was also performed. According to the alignment results (see Fig. S1B in the supplemental material) for the PhaC subunits of *Har. marismortui* and *A. vinosum* (PhaC_{Hm} and PhaC_{Av}, respectively) (16, 29), seven conserved amino acids (C143, C162, C190, D317, H318, H346, and W366) in *Har. marismortui* PhaC (see Fig. S1B) were selected for further investigation.

The corresponding amino acid residues of the putative catalytic triad C162-D317-H346 have been proved to be important for known bacterial PHA synthases (1, 9, 16, 17, 29). In haloarchaea, the replacement of C162 and D317 with Ala abolished the whole enzyme activity of PHA synthase *in vivo* (<2.5% of that for the wild-type enzyme), whereas the replacement of C162 or D317 with Ser or Asn resulted in an enzyme with a low level of activity (8% or 14.5% of that for the wild-type enzyme). Similarly, H346Q mutations resulted in inactivation of PHA synthase (Table 4). It is noteworthy that the conserved H318, which is adjacent to D317, was also proposed to be important in catalysis, and the H318Q mutation indeed reduced enzyme activity by half (Table 4). These results demonstrate that residues C162, D317, and H346 are likely also the catalytic triad of haloarchaeal PHA synthases.

However, some amino acid residues were found to be essential in haloarchaeal PhaC but not in PhaC_{Av}. For instance, the mutation of C130A in PhaC_{Av} could not affect the activity of PHA synthase (16). However, when the corresponding amino acid in PhaC_{Hm}, C143 (see Fig. S1B in the supplemental material), was subjected to mutagenesis analysis, the enzyme activity of the strain with C143S decreased slightly (80% of that for the wild-type enzyme), while that of the strain with C143A decreased largely (12.5% of that for the wild type). In the case of Cys190 in PhaC_{Hm}, although there was a different residue (Asp) in this position in PhaC_{Av} (see Fig. S1B), we found that the mutation of C190A in PhaC_{Hm} resulted in an inactive enzyme, illustrating that this residue was also essential for haloarchaeal PHA synthase activity. The conserved residue W366 was also mutated to Ala, which reduced the *in vivo* enzyme activity by half, whereas the corresponding residue of PhaC_{Av} has not yet been investigated. W366 might play an important role in protein-protein interaction, as was found for the respective amino acid residue of type I and II PHA synthases (1). In short, our mutagenesis studies showed that the haloarchaeal PHA synthase possessed some special key amino acids which were not found in the subtype IIIB PHA synthase from bacteria. It further confirms that the haloarchaeal PHA synthases from extremely saline environments have indeed diverged and evolved to form a novel subtype.

In conclusion, we report that scl-PHAs are produced by most haloarchaeal strains, especially in the presence of excess carbon substrates. A novel subtype of PHA synthase designated IIIA (A for halophilic *Archaea*), which is different from subtype IIIB (B for *Bacteria*) of *Bacteria*, is widely distributed in the *Halobacteriaceae* and appears to be involved in the biosynthesis of scl-PHAs. Comparison of the structure and function of these synthases would deepen our understanding of the evolution of PHA synthase, as well as their adaptation to different environments.

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