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Characterization of polyhydroxyalkanoate synthases from *Halomonas* sp. O-1 and *Halomonas elongata* DSM2581: Site-directed mutagenesis and recombinant expression

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are a group of microbial intracellular biopolyesters that have wide potential applications for the plastics industry. Here, we report the results of molecular analyses of PHA synthases from *Halomonas* species, which are halophilic eubacteria known as promising industrial PHA producers, but the PHA synthase gene of *Halomonas* has not been cloned previously. In this study, the putative PHA synthase genes (*phaC1* and *phaC2*) from two *Halomonas* strains (a new isolate, *Halomonas* sp. O-1, and the genome-sequenced strain, *Halomonas elongata* DSM2581) were cloned and characterized. We identified a gene related to the *H. elongata* DSM2581 PHA synthase gene ($phaC1_{He}$) in the *Halomonas* sp. O-1 genome ($phaC1_{HO1}$). PHA synthases usually contain a lipase box-like sequence Gly-X-Cys-X-Gly in their active sites. However, the equivalent sequence found in $PhaC1_{HO1}$ and $PhaC1_{He}$ was determined to be Ser-X-Cys-X-Gly, with serine replacing the first glycine. The cloned $phaC1_{HO1}$ and $phaC1_{He}$ genes were found to be functional when expressed in *Escherichia coli* JM109 and *Ralstonia eutropha* PHB^-4. Site-directed mutagenesis studies showed that the cysteine in the Ser-X-Cys-X-Gly sequence was the catalytic center of *Halomonas* PhaC1 proteins and that replacement of serine with glycine slightly affected PHA biosynthesis and copolymer composition. In addition, a second potential PHA synthase gene (*phaC2*) was cloned and its polymerization activity was evaluated. The results herein provide an important molecular basis for PHA production by *Halomonas* species.

1. Introduction

Polyhydroxyalkanoates (PHAs) are intracellular carbon-storage polymers that accumulate in bacteria and archaea. PHA can be used in biodegradable plastics manufacture because its physical properties resemble those of petroleum-based polyethylene and polypropylene [1–4]. PHA synthase encoded by the *phaC* gene catalyzes the final step of the PHA synthesis pathway, polymerizing (*R*)-3-hydroxyalkanoates into PHA. Thus, the nature of the synthase directly determines the mechanical properties of PHA products. PHA synthases have been classified into four classes based on substrate specificity and primary structure [5,6]. All previously characterized PHA synthases have a common catalytic center referred to as a lipase box-like sequence (Gly-X-Cys-X-Gly) [5], which is similar to the lipase box (Gly-X-Ser-X-Gly) in some lipases [7].

Halophiles are microorganisms that require salt (NaCl) to grow. They grow optimally at 5% salt or higher and can tolerate at least 10% salt [8]. The existence of PHA producing halophiles was first reported in a study by Kirk and Ginzburg in 1972, in which poly(3-hydroxylbutyrate) [P(3HB)] was extracted from an archaeon isolated from the Dead Sea [9]. From the 1980s through the 1990s, Rodriguez-Valera and colleagues studied PHA production by extremely halophilic archaea such as *Halobacterium* and *Haloferax* species [10–12]. A report on *Halomonas* by Quillaguamán et al., in 2005 was the first to study halophilic PHA-producing eubacteria [13]. *Halomonas* is a moderately halophilic eubacteria and has

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received increased attention as an industrial PHA producer for several reasons: 1) It utilizes diverse carbon sources. Some species can grow well with cheap and abundant carbon sources, such as starch hydrolysates [13] and glycerol, which is a by-product of biodiesel production [14]. 2) It produces a copolymer, poly(3HB-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], that possesses more favorable mechanical properties for plastic products than the P(3HB) homopolymer. To date, 2 *Halomonas* species, *Halomonas campisalis* [15] and *Halomonas profundus* [16], have been shown to produce P(3HB-*co*-3HV). 3) It is adaptable to stressful conditions. Most *Halomonas* species are also alkaline tolerant [17]. Recently, an arsenic tolerant *Halomonas* species was isolated from a saline soda lake [18]. These adaptabilities would be advantageous under severe industrial conditions. 4) Hypoosmotic shock may be used for PHA recovery. The cost of the current downstream process for PHA recovery accounts for 40% of the total production cost [19]. *Halomonas* may allow for the use of an easier and more cost effective PHA recovery method.

Halomonas has potential as an industrial PHA producer for many reasons, as mentioned above. In addition, it is known that PHA accumulation could play a crucial physiological role in the survival of hypersaline environments by *Halomonas* species. Cloning and characterization of PHA synthases or related genes in *Halomonas* species have not been conducted. However, *in silico* analyses suggested several candidates as related genes [20–22].

In this study, gene cloning and *in vivo* characterization of PHA synthase from halophilic eubacteria *Halomonas* were performed. Two *Halomonas* strains were compared, one of which was our own isolate from seawater, *Halomonas* sp. O-1. The other was *Halomonas elongata* DSM2581, which is a well-studied model strain that has been used for osmoregulation studies and genome analysis. The *phaC* from *Halomonas* sp. O-1 and *H. elongata* DSM2581 were expected to have a unique lipase box-like sequence (Ser-Tyr-Cys-Val-Gly) in their gene products, in which the first residue is conserved as glycine (Gly-X-Cys-X-Gly) in other PHA synthases. The heterologous expression of these *Halomonas phaC*s resulted in PHA accumulation in *Escherichia coli* and *Ralstonia eutropha* recombinants. These results provide valuable information for future applications of PHA synthase from *Halomonas* species.

2. Materials and methods

2.1. Isolation and characterization of Halomonas sp. O-1

Halomonas sp. O-1 was isolated from a seawater sample collected in the Fukuoka Prefecture of Japan. The isolation was carried out using lysogeny broth (LB) medium (yeast extract, 5 g/L; tryptone, 10 g/L; NaCl, 10 g/L) agar plates with 20 g/L glucose and 0.25 mg/mL Nile red to screen for PHA accumulating bacteria as previously described [23]. The PHA accumulating bacteria were isolated and genomic DNA was extracted using the phenol-chloroform method [24]. 16S rRNA was amplified using the forward primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1525R (5′-AAA GGA GGT GAT CCA GCC-3′). The PCR product was purified using a nucleotide purification kit (Qiagen GmbH, Hilden, Germany). DNA sequencing was performed by Hokkaido System Science Company (Sapporo, Japan). Sequence analysis and multiple alignment were performed using the BLAST web server. The complete *Halomonas* sp. O-1 16S rRNA sequence was submitted to the DDBJ database (accession number AB894359). Carbon source preference, morphology, gram stain, and motility testing was conducted. The API 50 CH kit (bioMérieux, Lyon, France) was used to characterize the carbon source metabolism of *Halomonas* sp. O-1. Growth was visually determined. PHA accumulation by *Halomonas* sp. O-1 using various carbon sources as the sole carbon source was determined after conversion to crotonic acid in H_2SO_4 as previously described [25]. PHA accumulation was also observed using fluorescence microscopy (Eclipse 80i, Nikon, Tokyo, Japan). Cells grown for 24 h on LB agar plates with Nile red were fixed on a glass slide for microscopic examination. Nile red fluorescence was viewed using 540 nm excitation and 605 nm emission filters (TRITC).

2.2. Cloning of Halomonas sp. O-1 and H. elongata DSM2581 phaC1 genes

To evaluate PHA synthases from *Halomonas* species, *phaC1* genes from *Halomonas* sp. O-1 and *H. elongata* DSM2581 (HELO_3394) were cloned. The primers $PhaC1_{HO1}$ F (5′-TCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT AAT GCC GTC TCA ACA C-3′) and $PhaC1_{HO1}$R (5′-TTG GTT GGA TCC CTA AGT ACT GCT GGG GAT TTC TCC TAA-3′) for *Halomonas* sp. O-1 and $PhaC1_{He}$F (5′-GCG CAT ATG ATG CAG CCA GGG AAT CAT GCA-3) and $PhaC1_{He}$R (5′-AAT GGA TCC CTA ACT CCC GGC TTC GCC-3′) for *H. elongata* DSM2581 were designed based on the respective nucleotide sequences of *H. elongata* DSM2581 (accession number NC_014532) genome. The PCR products were embedded into the pET3a cloning vector (Novagen, Madison, WI, USA). DNA sequencing was performed using three or more independent PCR products to obtain an error-free *phaC1* sequence. Each pET3a vector containing error-free *phaC1* genes was transformed to *E. coli* JM109. The sequence of the *phaC1* gene from *Halomonas* sp. O-1 has been deposited in DDBJ database (accession number AB920910).

2.3. Site-directed mutagenesis and plasmid construction

Site-directed mutagenesis using a commercial kit (GeneArt Site-Directed Mutagenesis System; Invitrogen, Carlsbad, CA, USA) was performed to investigate activity related to the lipase box-like sequence (Ser-Tyr-Cys-Val-Gly) of *Halomonas* sp. O-1 and *H. elongata* DSM2581. The first site-directed mutagenesis was to substitute an alanine for the cysteine residue at the Cys329 and Cys331 positions in *Halomonas* sp. O-1 and *H. elongata* DSM2581, respectively. The second site-directed mutagenesis was to substitute a glycine for the serine residue at positions Ser327 and Ser329 in *Halomonas* sp. O-1 and *H. elongata* DSM2581, respectively. The second mutation was designed to compare the wild-type sequence (Ser-Tyr-Cys-Val-Gly) with the common sequence (Gly-X-Cys-X-Gly) [5] found in other PHA synthases. The primers used for site-directed mutagenesis are listed in Table 1. The resulting pET3a plasmid with the mutated gene was digested using *Xba*I and *Bam*HI. The *phaC1* fragment was ligated into pGEM″ABex [26], which harbors the *phaA* and *phaB* genes from *R. eutropha* H16, encoding for β-ketothiolase and NADPH-dependent acetoacetyl-CoA reductase, respectively. The resulting plasmid containing the *phaC1AB* gene cluster was transformed into *E. coli* JM109 for analysis of PHA accumulation and molecular weights. Expression of the pGEM″-PhaC1AB plasmid in *R. eutropha* was required to investigate the ability of the introduced PhaC1 to produce copolymers. The *phaC1AB* gene cluster was excised with *Bam*HI and ligated into pBBRMCS-2 [27] with an *R. eutropha* promoter for expression in *R. eutropha* PHB$^-$4. Plasmid transformation into *R. eutropha* PHB$^-$4 was performed using *E. coli* S17-1 [28] as a plasmid donor for inter-generic plasmid conjugations.

2.4. Expression of Halomonas phaC2 in recombinant E. coli JM109

The PHA synthase candidate gene *phaC2* was cloned. The sequence of *phaC2* from *Halomonas* sp. O-1 has been deposited in DDBJ database (accession number AB920911). To further characterize this gene, *phaC2* from *Halomonas* sp. O-1 and *H. elongata* DSM2581

Table 1
PCR primers used for *phaC1* site-directed mutagenesis.

PHA synthase	Primer	Primer direction	Amino acid substitution	Sequence[a]
$PhaC1_{HO1}$	fC329A	Forward	Cys-329 to Ala	5′-CTGCTGAGCTACGCCGTGGGCGGCACGCTG-3′
	rC329A	Reverse	Cys-329 to Ala	5′-CGTGCCGCCCACGGCGTAGCTCAGCAGGTT-3′
	fS327G	Forward	Ser-327 to Gly	5′-GTCAACCTGCTGGGCTACTGCGTGGGCGGC-3′
	fS327G	Reverse	Ser-327 to Gly	5′-GCCCACGCAGTAGCCCAGCAGGTTGACCGA-3′
$PhaC1_{He}$	fC331A	Forward	Cys-331 to Ala	5′-CTGCTGAGCTACGCCGTCGGCGGGACTCTG-3′
	rC331A	Reverse	Cys-331 to Ala	5′-AGTCCCGCCGACGGCGTAGCTCAGCAGGTT-3′
	fS329G	Forward	Ser-329 to Gly	5′-GTCAACCTGCTGGGCTACTGTGTCGGCGGG-3′
	rS329G	Reverse	Ser-329 to Gly	5′-GCCGACACAGTAGCCCAGCAGGTTGACCGA-3′

[a] Underlined sequences are the mutation sites.

(HELO_2207) was subcloned into pGEM″ABex [26] using the primers $PhaC2_{HO1}$F (5′-GGC TTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT AAT GAA CTT TCT CGC AAA C-3′) and $PhaC2_{HO1}$R (5′-A CT CGG CAA CCC AAA TAG AAG CTT CCT ATA-3′) for *Halomonas* sp. O-1, and $PhaC2_{He}$F (5′-TCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT AAT GTA TGC CAT GAT G-3′) and $PhaC2_{He}$R (5′-C CG AGG ACG ATG AAG CTT ACA AGC GAA-3′) for *H. elongata* DSM2581. The resulting plasmid was then transformed into *E. coli* JM109 and stained with Nile red on LB agar plates to visually examine PHA accumulation as previously described [23].

2.5. Bacterial strains and culture conditions for PHA accumulation

The strains and plasmids used for PHA accumulation are listed in Table 2. PHA accumulation in *Halomonas* sp. O-1 and *H. elongata* DSM2581 was determined under shake-flask cultivation conditions. Each *Halomonas* strain was cultured in shake-flasks containing 100 mL LB medium (yeast extract, 5 g/L; tryptone, 10 g/L; NaCl 10 g/L) and 20 g/L glucose with or without organic acids. The strain was cultivated in a reciprocal shaker at 130 rpm for 72 h at 30 °C. For determination of carbon source utilization by *Halomonas* sp. O-1, M9 minimal medium ($Na_2HPO_4{\cdot}7H_2O$, 12.8 g/L; KH_2PO_4, 3.0 g/L; NH_4Cl, 1.0 g/L; $MgSO_4{\cdot}7H_2O$, 2 mM; $CaCl_2{\cdot}2H_2O$, 0.1 mM) with 2.5 g/L NaCl and 20 g/L of the indicated carbon compounds as the sole carbon source was used. The strain was cultivated in 10 mL of M9 medium in a test tube with shaking at 100 rpm for 24–48 h at 30 °C. Recombinant *E. coli* JM109 was cultured in shake-flasks containing 100 mL LB medium with 20 g/L glucose and 100 mg/L ampicillin for 72 h at 37 °C. Recombinant *R. eutropha* PHB^-4 was cultured in minimal salt (MS) medium ($Na_2HPO_4{\cdot}12H_2O$, 9 g/L; KH_2PO_4, 1.5 g/L; NH_4Cl, 0.5 g/L; $MgSO_4{\cdot}7H_2O$, 0.2 g/L; trace metal element solution) [29] with 100 mg/L kanamycin. Cultivation was carried out in shake-flasks containing 100 mL MS medium in a reciprocal shaker at 130 rpm for 72 h at 30 °C. The carbon sources (sodium butyrate, sodium valerate, and sodium hexanoate) were injected at a final concentration of 0.5 g/L 5 times every 12 h over the course of 72 h to minimize growth retardation. All flask incubation was done in triplicate to ensure data accuracy.

2.6. PHA analyses

High performance liquid chromatography (HPLC) was used to determine crotonic acid concentration in cell hydrolysate correspond to PHA of *Halomonas* sp. O-1 cultured with various carbon sources [25]. The HPLC samples were prepared as follows: 500 μL of culture in M9 minimal medium was incubated with 2.5 g/L NaCl for 24–48 h. One milliliter of H_2SO_4 was added, and the mixture was incubated at 120 °C for 40 min. Four milliliters of 0.014 N H_2SO_4 was added and the mixture was filtered (pore size, 0.45 μm; Milex-LH PTFE, Merck Millipore, Darmstadt, Germany). The sample was

Table 2
Strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference
Strains		
Halomonas sp. O-1	Isolated from seawater in Japan	This study
Halomonas elongata	Obtained from DSMZ	$DSM2581^T$
Escherichia coli JM109	PHA synthase expression host	TaKaRa Bio.
Escherichia coli S17-1	Plasmid donor strain for intergeneric conjugation with *R. eutropha* strain	[28]
Ralstonia eutropha PHB-4	Non PHA-accumulating mutant, recombinant expression host	DSM541
Plasmids		
pET3a	T7 promoter, *Nde*I and *Bam*HI cloning site, Amp^r	Novagen
pET3a″$PhaC_{HO1}$	Cloning vector for site-directed mutagenesis, PhaC1 *Halomonas* sp. O-1	This study
pET3a″$PhaC_{He}$	Cloning vector for site-directed mutagenesis, PhaC1 *Halomonas elongata*	This study
pGEM″ABex	pGEM-T derivative; pha_{Re} promoter, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	[26]
pGEM″$PhaC1_{HO1}$AB_WT	pGEM″ABex derivative, *phaC1* from *Halomonas* sp. O-1, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC1_{HO1}$AB_CA	pGEM″ABex derivative, *phaC1* from *Halomonas* sp. O-1 C329A mutant, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC1_{HO1}$AB_SG	pGEM″ABex derivative, *phaC1* from *Halomonas* sp. O-1 S327G mutant, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC1_{He}$AB_WT	pGEM″ABex derivative, *phaC1* from *H. elongata*, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC1_{He}$AB_CA	pGEM″ABex derivative, *phaC1* from *H. elongata* C331A mutant, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC1_{He}$AB_SG	pGEM″ABex derivative, *phaC1* from *H. elongata* S329G mutant, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC2_{HO1}$AB	pGEM″ABex derivative, *phaC2* from *Halomonas* sp. O-1, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM″$PhaC2_{He}$AB	pGEM″ABex derivative, *phaC2* from *H. elongata*, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pGEM"PhaC2C1$_{HO1}$AB	pGEM″ABex derivative, *phaC2* and *phaC1* from *Halomonas* sp. O-1, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Amp^r	This study
pBBR1MCS-2	Broad-host-range cloning vector, Kan^r	[27]
pBBR1$PhaC1_{HO1}$AB_WT	pBBR1MCS-2 derivative, *phaC1* from *Halomonas* sp. O-1, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Kan^r	This study
pBBR1$PhaC1_{HO1}$AB_SG	pBBR1MCS-2 derivative, *phaC1* from *Halomonas* sp. O-1 S327G mutant, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Kan^r	This study
pBBR1$PhaC1_{He}$AB_WT	pBBR1MCS-2 derivative, *phaC1* from *H. elongata*, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Kan^r	This study
pBBR1$PhaC1_{He}$AB_SG	pBBR1MCS-2 derivative, *phaC1* from *H. elongata* S329G mutant, $phaA_{Re}$ and $phaB_{Re}$ from *R. eutropha* H16, Kan^r	This study

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

analyzed using a Waters 600S HPLC system (Waters Corporation, Milford, MA) with an Aminex Fermentation Monitoring Column (Bio-Rad Laboratories, Hercules, CA) and a Waters 486 UV detector (Waters). The column oven temperature was 60 °C. The mobile phase was 0.014 N H_2SO_4, with a flow rate of 0.7 mL/min.

Gas chromatography (GC) was performed to determine the PHA content and composition of cells collected from shake-flasks after lyophilization. GC samples were prepared by methanolysis using 15% v/v sulfuric acid as previously described [29] and analyzed using a Shimadzu GC-14D system with a flame ionization detector (Shimadzu, Kyoto, Japan). The initial temperature was 100 °C for 1 min and then increased to 280 °C in continuous steps of 8 °C/min.

The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of extracted PHA from collected biomass were measured using gel permeation chromatography (GPC) with a Shimadzu 10A GPC system (Shimadzu) equipped with a 10A refractive index detector and two K-806M joint columns (Shodex, Tokyo, Japan). The column oven temperature was 40 °C, and low polydispersity polystyrene was used as a standard. Chloroform was used as the mobile phase with a flow rate of 0.8 mL/min. Purified PHA samples extracted from each biomass obtained from shake-flasks were dissolved in chloroform and injected at a concentration of 1.0 mg/mL.

3. Results and discussion

3.1. Isolation and characterization of Halomonas sp. O-1

Halomonas sp. O-1 isolated from seawater was found to be a gram-negative, obligate aerobic, motile, rod-shaped bacterium with a length of 5–15 μm. Microscopic images of the bacteria are shown in Fig. 1. *Halomonas* sp. O-1 grew well at NaCl concentrations of 5–15% with an optimum concentration of 10%. Growth was optimal at a pH of 6.0–9.5. Catalase and oxidase tests were positive. The sequence of the 16S rRNA gene (1526 bp) was closely related to that of *Halomonas* sp. MAN K22 with 98% similarity. The carbon source preference of *Halomonas* sp. O-1 is summarized in Table 3. PHA accumulation in *Halomonas* sp. O-1 was most robust with glucose, pectin, or glycerol as carbon sources. Further taxonomic characterization is required for more precise identification at the species level [17].

3.2. Cloning of Halomonas sp. O-1 and H. elongata DSM2581 phaC1 genes

Analysis of *phaC1* genes from *Halomonas* sp. O-1 and *H. elongata* DSM2581 showed that these PHA synthases are consistent with class I synthases and have a lipase box-like sequence (Ser-X-Cys-X-Gly) as shown in Fig. 2. The serine in this sequence is unique and found in *Halomonas* PhaC1 proteins as deduced from genome databases. *Halomonas* sp. O-1 and *H. elongata* DSM2581 PHA synthase operons have a structure of *phaP1P2C*, which is quite different from the *phaCAB* structure found in the representative class I synthase operons from *R. eutropha* [20]. The genes *phaP1* and *phaP2* encode phasin proteins, which are structural proteins influencing the PHA granule surface-to-volume ratio [30]. An additional candidate PHA synthase gene was found in *Halomonas* genomes [20] that is referred to as *phaC2* and is addressed below in detail. These previously unknown features of *Halomonas phaC1* and its related gene candidates led us to conduct cloning of its *phaC* genes to verify their activity.

Table 3
Growth and PHA accumulation in *Halomonas* sp. O-1 with various carbon sources.

Carbon source	Growth	PHA accumulation[a]
D-Glucose	+++	+
Sucrose	++	+
D-Xylose	+	+
Trehalose	+	+
Starch	++	+
D-Galactose	+	+
Pectin	++	+
D-Fructose	+	ND
Glycerol	+[b]	+
Maltose	+[b]	ND
D-Turanose	+[b]	ND
D-Fucose	+[b]	ND
Lactose	+	ND
Cellulose	+	ND
Chitin	+	ND
Alginate	+	ND

ND, not determined.
[a] PHA was measured as crotonic acid in H_2SO_4 [25].
[b] Tested using an API 50 CH kit.

3.3. PHA accumulation by Halomonas sp. O-1 and H. elongata DSM2581

Shake-flask cultivation was carried out for PHA accumulation in *Halomonas* sp. O-1 and *H. elongata* DSM2581 (Table 4). *Halomonas* sp. O-1 grew with a dry cell weight of 6.9 ± 0.7 g/L and a concentration of accumulated P(3HB) of up to 31 ± 7 wt% after 72 h using glucose as the sole carbon source. When sodium valerate was added, the dry cell weight of *Halomonas* sp. O-1 reached 7.6 ± 0.3 g/L and P(3HB-*co*-3HV) was produced at a content of 28 ± 1 wt%. *H. elongata* DSM2581 grew and produced P(3HB) in the similar

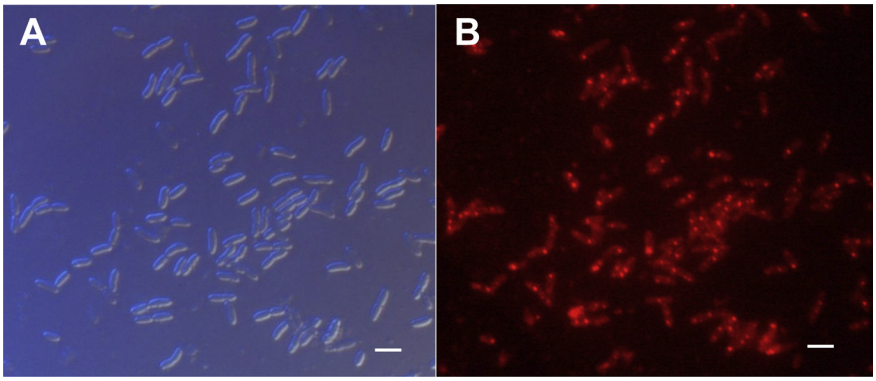

Fig. 1. Microscopic images of *Halomonas* sp. O-1 viewed under 1000× magnification. (A) Light-field image. (B) Fluorescent image. Nile red fluorescence of PHA was viewed using 540 nm excitation and 605 nm emission filters (TRITC). Scale bar: 5 μm.

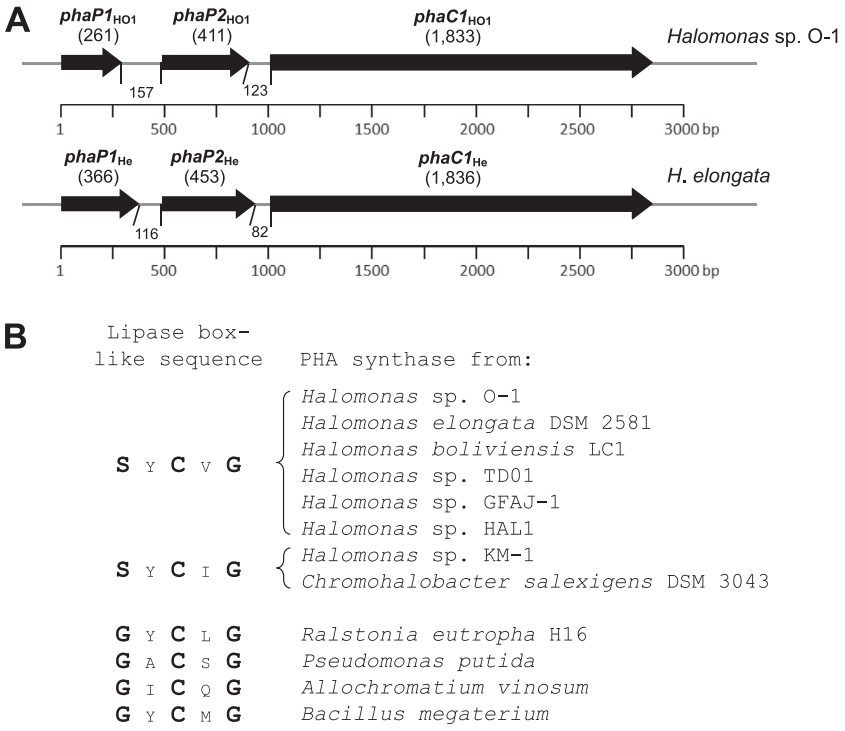

Fig. 2. (A) PHA operons of *Halomonas* sp. O-1 (top row) and *H. elongata* DSM2581 (bottom row) with detailed ORFs. (B) Lipase box-like sequences from various bacteria and *Halomonas* species.

manner when glucose was the sole carbon source, whereas it was not able to grow using sodium valerate. This result contrasts with the sodium valerate tolerance of *Halomonas* sp. O-1. Both strains experienced growth retardation when sodium hexanoate was added to the medium. This growth inhibition by sodium alkanoate has been well documented [31]. Two species in the genus *Halomonas* have been reported as copolymer producers. *H. campisalis* was found to produce P(3HB-*co*-3HV) without any precursor [15], whereas *H. profundus* produced the same copolymer with sodium valerate as a precursor [16] as did *Halomonas* sp. O-1. The weight-average molecular weight (M_w) values of P(3HB) produced by *Halomonas* sp. O-1 and *H. elongata* DSM2581 were 4.6×10^5 and 3.9×10^5, respectively, which are lower than that of *H. campisalis* MCM B-1027 (13.0×10^5) [15].

3.4. PHA accumulation by recombinant E. coli expressing PhaC1 proteins

The purpose of expressing PhaC1 derived from *Halomonas* sp. O-1 and *H. elongata* DSM2581 in recombinant *E. coli* JM109 was to validate synthase activity and the catalytic center of each *Halomonas* PhaC1 protein. The secondary purpose was to observe the effect of altering the serine residue of the PhaC1 lipase box-like sequence to glycine. Shake-flask cultivation with recombinant *E. coli* JM109 harboring each plasmid was carried out (Table 5). The P(3HB) content resulting from the expression of PhaC1 from *Halomonas* sp. O-1 in *E. coli* was approximately twice that of *H. elongata* DSM2581. The substitution of alanine for Cys329 and Cys331 of the lipase box-like sequence resulted in complete loss of PHA accumulation in recombinant *E. coli* JM109. It has been reported that mutation of the central cysteine of the lipase box-like sequence in $PhaC1_{Re}$ from *R. eutropha* resulted in complete loss of PHA synthase activity [32]. These results suggested that Cys329 and Cys331 were the catalytic cysteines in these PhaC1 proteins. The substituting a glycine for the serine in the lipase box-like sequence in both PhaC1 proteins did not seem to influence growth or P(3HB) content but slightly increased the weight-average molecular weight (M_w). The recombinant *E. coli* in this study did not possess any PHA depolymerase. Therefore, the M_w of generated P(3HB) might reflect a change in the catalytic activity of PhaC1 induced by the substitution.

Table 4
Shake-flask cultivation of *Halomonas* sp. O-1 and *H. elongata* DSM2581 using glucose and organic acids.[a]

Strain	Carbon source[b]	Dry cell weight (g/L)	PHA content (wt%)	PHA composition (mol%)		Molecular weight[c]		
				3HB	3HV	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
Halomonas sp. O-1	Glucose	6.9 ± 0.7	31 ± 7	100	–	1.6	4.6	2.9
	Glucose + C5	7.6 ± 0.3	28 ± 1	72 ± 1	28 ± 1	–	–	–
	Glucose + C6	0.6 ± 0.1	ND	–	–	–	–	–
H. elongata DSM2581	Glucose	6.6 ± 0.4	32 ± 7	100	–	2.2	3.9	1.8
	Glucose + C5	0.7 ± 0.1	ND	–	–	–	–	–
	Glucose + C6	0.5 ± 0.1	ND	–	–	–	–	–

ND, not detected.
[a] Cells were cultured in LB medium containing glucose (20 g/L) with or without organic acid (injection of 0.5 g/L 5 times every 12 h totaling 2.5 g/L to minimize toxicity) at 30 °C for 72 h.
[b] C5, sodium valerate; C6, sodium hexanoate.
[c] M_n, number-average molecular weight; M_w, weight-average molecular weight.

Table 5
Shake-flask cultivation of recombinant *E. coli* JM109 expressing PhaC1 from *Halomonas* sp. O-1 and *H. elongata* DSM2581.[a]

Expressed PHA synthase	Dry cell weight (g/L)	P(3HB)[b] content (wt%)	Molecular weight[c]		
			M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
$PhaC1_{HO1}$ WT	3.6 ± 0.3	40 ± 5	15	20	1.3
$PhaC1_{HO1}$ S327G	2.9 ± 0.8	46 ± 4	13	27	2.0
$PhaC1_{HO1}$ C329A	1.8 ± 0.9	ND	–	–	–
$PhaC1_{He}$ WT	3.0 ± 0.7	23 ± 1	11	20	1.9
$PhaC1_{He}$ S329G	2.8 ± 0.6	20 ± 1	14	27	1.9
$PhaC1_{He}$ C331A	1.9 ± 0.2	ND	–	–	–

ND, not detected.
[a] Cells were cultured in LB medium containing glucose (20 g/L) at 37 °C for 72 h.
[b] P(3HB), poly(3-hydroxybutyrate).
[c] M_n, number-average molecular weight; M_w, weight-average molecular weight.

3.5. PHA accumulation by recombinant *R. eutropha* PHB⁻4 expressing PhaC1

PhaC1 proteins derived from *Halomonas* sp. O-1 and *H. elongata* DSM2581 were expressed in *R. eutropha* PHB⁻4 to examine 3HA monomers available for PHA synthesis. *R. eutropha* has the ability to produce 3HB, 3HV, and 3-hydroxyhexanoate (3HHx) from butyrate, valerate, and hexanoate, respectively [33]. Four types of recombinant *R. eutropha* PHB⁻4 were cultivated in MS medium with organic acids as the sole carbon source as shown in Table 6. Cultivation of recombinant *R. eutropha* PHB⁻4 in MS medium supplied with sodium butyrate and hexanoate as the sole carbon source resulted in the accumulation of P(3HB) homopolymer. The P(3HB) content resulting from wild-type and mutant PhaC1 appeared to correlate with the increase in dry cell weight. When sodium valerate was supplied in the medium, 3HV generation altered the PHA composition with PhaC1 from both *Halomonas* sp. O-1 and *H. elongata* DSM2581. This result indicated that PhaC1 proteins from these two strains have affinity for 3HV monomers although the original strain of *H. elongata* DSM2581 did not produce P(3HB-*co*-3HV) from sodium valerate and the amino acid sequence homology between PhaC1 proteins of *Halomonas* sp. O-1 and *H. elongata* DSM2581 was relatively low (73%). This result also suggested that other *Halomonas* derived PhaC1 proteins may have affinity for 3HV. Recombinant *R. eutropha* grew using sodium hexanoate as the sole carbon source, but with only trace amounts of 3HHx when expressing PhaC1 proteins from these two strains, suggesting that PhaC1 proteins from *Halomonas* sp. O-1 and *H. elongata* DSM2581 did not have affinity for 3HHx.

3.6. Expression of *Halomonas phaC2* in recombinant *E. coli*

Another PHA synthase candidate gene, which is referred to as *phaC2*, was found in *Halomonas* sp. O-1 and *H. elongata* DSM2581 genomes. Products of this gene from both species were expected to have a Gly-Asn-Cys-Gln-Ala sequence in their putative active site [20], which is slightly different than the lipase box-like sequence found in PHA synthases. To evaluate the polymerization activity of these *phaC2* products, *phaC2* genes from *Halomonas* sp. O-1 and *H. elongata* DSM2581 were expressed in recombinant *E. coli* JM109 along with $phaA_{Re}$ and $phaB_{Re}$. Recombinant *E. coli* JM109 was streaked on glucose-containing LB agar plates with 0.25 mg/L Nile red, and PHA accumulation was observed as shown in Fig. 3. Expression of *phaC2* in recombinant *E. coli* JM109 did not lead to PHA accumulation. Then, we constructed the plasmid with *phaC1* and *phaC2* for the co-expression. The *phaC2* was amplified from the pET3a-cloned *phaC2* with a set of primers that included the SD sequence of the plasmid and were modified with the terminals to be cleaved by *Xba*I. The *phaC2* amplicon was inserted into the *Xba*I site on the pGEM"$PhaC1_{HO1}$AB_WT. The resulting order of the genes on the plasmid was *phaC2*, *phaC1*, *phaA* and *phaB*. However, co-expression of *Halomonas* sp. O-1 *phaC1* and *phaC2* resulted in similar fluorescence intensity to that resulting from expression of *phaC1* alone. This observation is in contrast to the characteristics of *phaC* gene regulation in *Bradyrhizobium japonicum* USDA110, a bacterium in which the expression of *phaC2* resulted in decreased PHA production, presumably due to inhibition of PhaC1 activity by PhaC2 [34]. It was concluded from this result that *phaC2* expression was not responsible for PHA synthesis in the recombinant *E. coli* JM109. Taken together, our observations suggest that *phaC1* is the

Table 6
Shake-flask cultivation of recombinant *R. eutropha* PHB⁻4 cells expressing PhaC1 from *Halomonas* sp. O-1 and *H. elongata* DSM2581.[a]

Expressed PhaC1	Carbon source[b]	Dry cell weight (g/L)	PHA content (wt%)	PHA composition (mol%)		
				3HB	3HV	3HHx
$PhaC1_{HO1}$ WT	C4	1.1 ± 0.4	46 ± 5	100	–	–
	C5	1.2 ± 0.6	45 ± 2	42 ± 1	58 ± 1	–
	C6	1.3 ± 0.2	56 ± 2	100	–	Trace[c]
$PhaC1_{HO1}$ S327G	C4	1.2 ± 0.4	51 ± 3	100	–	–
	C5	1.0 ± 0.1	35 ± 9	63 ± 1	37 ± 1	–
	C6	1.5 ± 0.1	56 ± 2	100	–	Trace[c]
$PhaC1_{He}$ WT	C4	0.8 ± 0.1	33 ± 1	100	–	–
	C5	0.8 ± 0.1	21 ± 2	77 ± 1	23 ± 1	–
	C6	0.9 ± 0.1	34 ± 2	100	–	Trace[c]
$PhaC1_{He}$ S329G	C4	1.1 ± 0.4	48 ± 3	100	–	–
	C5	1.1 ± 0.1	30 ± 1	59 ± 1	41 ± 1	–
	C6	1.3 ± 0.4	52 ± 3	100	–	Trace[c]

[a] Cells were cultured in mineral salt (MS) medium containing C4, C5, or C6 sodium alkanoates (injection of 0.5 g/L 5 times every 12 h totaling 2.5 g/L to minimize toxicity) as the sole carbon source at 30 °C for 72 h.
[b] C4, sodium butyrate; C5, sodium valerate; C6, sodium hexanoate.
[c] The detected amount was less than 1 mol%.

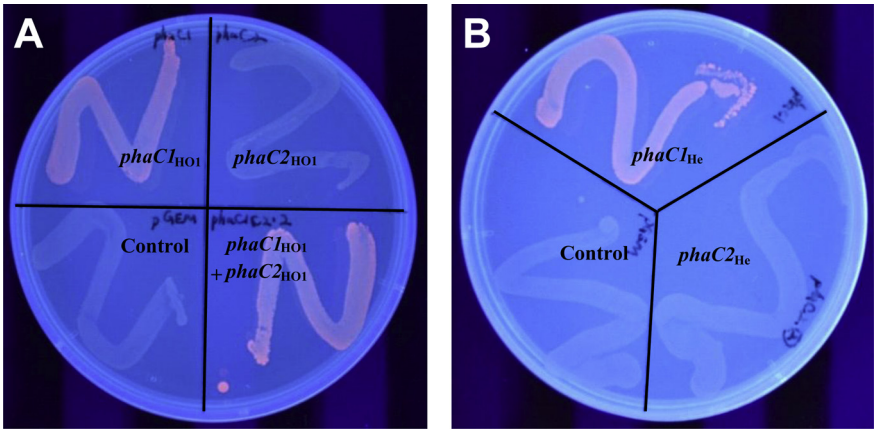

Fig. 3. Illuminated LB plates with Nile red viewed under UV light. (A) Recombinant *E. coli* JM109 expressing PhaC1, PhaC2, or both PhaC1 and PhaC2 from *Halomonas* sp. O-1. (B) Recombinant *E. coli* JM109 expressing PhaC1 or PhaC2 from *H. elongata* DSM2581. Recombinant *E. coli* JM109 with pGEM″ABex and without PhaC1 was used as a control.

primary functional gene encoding PHA synthase in the original strains of *Halomonas* sp. O-1 and *H. elongata* DSM2581.

4. Conclusions

In this study, the PHA synthase genes of *Halomonas* sp. O-1 and *H. elongata* DSM2581 were cloned and heterologously expressed in *E. coli* JM109 and *R. eutropha* PHB$^-$4. It was found that both PhaC1 proteins expressed in these two strains exhibited PHA synthase activity in *E. coli* and produced P(3HB-*co*-3HV) in *R. eutropha* from valerate, although *H. elongata* DSM2581 was not able to grow on valerate. Site-directed mutagenesis of a potential lipase box-like sequence (Ser-Tyr-Cys-Val-Gly) found in the PhaC1 revealed that the central cysteine in the sequence was critical for catalytic activity and could be functional as the catalytic center. The PhaC1 mutant with a substitution of glycine for serine retained its activity. Another candidate PHA synthase gene (*phaC2*) was also cloned and analyzed; however, a recombinant *E. coli* expressing this gene did not accumulate PHA. Overall, this study provides basic and useful information for future studies on PHA production by *Halomonas* and other halophiles.

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