

# Identification of the Polyhydroxyalkanoate (PHA)-Specific Acetoacetyl Coenzyme A Reductase among Multiple FabG Paralogs in *Haloarcula hispanica* and Reconstruction of the PHA Biosynthetic Pathway in *Haloferax volcanii*<sup>∇†</sup>

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**Genome-wide analysis has revealed abundant FabG ( $\beta$ -ketoacyl-ACP reductase) paralogs, with uncharacterized biological functions, in several halophilic archaea. In this study, we identified for the first time that the *fabG1* gene, but not the other five *fabG* paralogs, encodes the polyhydroxyalkanoate (PHA)-specific acetoacetyl coenzyme A (acetoacetyl-CoA) reductase in *Haloarcula hispanica*. Although all of the paralogous *fabG* genes were actively transcribed, only disruption or knockout of *fabG1* abolished PHA synthesis, and complementation of the  $\Delta$ *fabG1* mutant with the *fabG1* gene restored both PHA synthesis capability and the NADPH-dependent acetoacetyl-CoA reductase activity. In addition, heterologous coexpression of the PHA synthase genes (*phaEC*) together with *fabG1*, but not its five paralogs, reconstructed the PHA biosynthetic pathway in *Haloferax volcanii*, a PHA-defective haloarchaeon. Taken together, our results indicate that FabG1 in *H. hispanica*, and possibly its counterpart in *Haloarcula marismortui*, has evolved the distinct function of supplying precursors for PHA biosynthesis, like PhaB in bacteria. Hence, we suggest the renaming of FabG1 in both genomes as PhaB, the PHA-specific acetoacetyl-CoA reductase of halophilic archaea.**

Several haloarchaeal species belonging to the genera *Haloferax*, *Haloarcula*, *Natrialba*, and *Haloquadratum* are capable of synthesizing short-chain-length polyhydroxyalkanoates (SCL-PHAs) (6, 8, 14, 16), a large family of biopolymers with desirable biodegradability, biocompatibility, and thermoplastic features (31). Although the metabolic pathways of PHAs in bacteria have been characterized in detail (10, 15, 20, 25, 26, 37), the genes involved in PHA biosynthesis in haloarchaea were not recognized until recently, when the PHA synthase genes were identified and characterized for *Haloarcula marismortui*, *Haloarcula hispanica*, and *Haloferax mediterranei* (6, 19). These archaeal PHA synthases are all composed of two subunits, PhaE and PhaC. They are homologous to the class III PHA synthases from bacteria but have a longer C-terminal extension in the PhaC subunit. Nevertheless, the pathway of supplying the PHA precursors has not yet been clarified for any haloarchaeal strain.

Both *H. mediterranei* and *H. hispanica* are able to synthesize poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from unrelated carbon sources, despite the content of the (*R*)-3-hydroxyvalerate (3-HV) monomer of PHBV in *H. mediterranei* (10 to 13 mol%) (4, 19) being much higher than that in *H. hispanica* (~3 mol%) (19). Conversely, the bacteria *Ralstonia*

*eutropha* and *Synechocystis* sp. strain PCC6803, which possess class I and III PHA synthases, respectively, accumulate just poly(3-hydroxybutyrate) (PHB) when the 3-HV-related carbon sources (i.e., propionate and valerate) are not supplied (30). In these two bacteria, the biosynthesis of the (*R*)-3-hydroxybutyrate coenzyme A [(*R*)-3-HB-CoA] precursor is conducted by two steps. First, two acetyl-CoA molecules are condensed into one acetoacetyl-CoA molecule by the enzyme  $\beta$ -ketothiolase (PhaA). The acetoacetyl-CoA is then reduced to (*R*)-3-HB-CoA by a PHA-specific acetoacetyl-CoA reductase (PhaB). The resulting (*R*)-3-HB-CoA is subsequently incorporated into PHB, catalyzed by PHA synthases (26, 36).

Both PhaB and FabG belong to the short-chain dehydrogenase/reductase (SDR) superfamily, whose members are homologous in sequence and have several conserved motifs (27, 29). Interestingly, although FabGs naturally reduce 3-ketoacyl-ACP to form (*R*)-3-hydroxyacyl-ACP in fatty acid biosynthesis, a few FabGs also recognize 3-ketoacyl-CoA and hence function in PHA biosynthesis. For example, the FabG proteins of *Escherichia coli* and *Pseudomonas aeruginosa* have been demonstrated to supply precursors for PHA biosynthesis in recombinant *E. coli* cells (21, 22, 32, 35). In addition, several FabG paralogs may have evolved a distinct function, to be responsible only for PHA accumulation. This situation was observed in *Synechocystis* sp. strain PCC6803, where the originally annotated FabG (12) was renamed PhaB after an understanding of its function in PHA biosynthesis (36).

Genome-wide analysis of *H. marismortui* ATCC 43049 (1) revealed eight FabG paralogs in this haloarchaeon. Similarly, multiple *fabG* paralog genes (*fabG1* to *fabG6*) were also observed in the newly sequenced genome of *H. hispanica* (our unpublished data). In this study, we demonstrate that *fabG1*, but not the other five *fabG* paralogs, encodes the PHA-specific

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<b>Strains</b>		
<i>H. hispanica</i> AS2049	Wild-type strain (CGMCC1.2049 or ATCC 33960)	China General Microbiological Culture Collection Center
<i>H. hispanica</i> $\Delta$ <i>fabG1</i>	<i>fabG1</i> -deleted mutant of <i>H. hispanica</i> via double-crossover recombination	This study
<i>H. volcanii</i> DS70	Wild-type strain cured of endogenous pHV2 plasmid	39
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i>	33
<b>Plasmids</b>		
pWL102	10.5-kb shuttle vector; Amp <sup>r</sup> Mev <sup>r</sup>	13
pL52	Derivative of pWL102 containing the <i>hsp-5</i> promoter of <i>Halobacterium salinarium</i>	18
pWLG1	Derivative of pL52, 11.2 kb, <i>fabG1</i>	This study
pWLEC	Derivative of pWL102, 12.6 kb, <i>phaEC</i> and native promoter	This study
pWLG1EC	Derivative of pL52, 13.4 kb, <i>fabG1</i> and <i>phaEC</i>	This study
pWLG2EC	Derivative of pL52, 13.3 kb, <i>fabG2</i> and <i>phaEC</i>	This study
pWLG3EC	Derivative of pL52, 13.4 kb, <i>fabG3</i> and <i>phaEC</i>	This study
pWLG4EC	Derivative of pL52, 13.4 kb, <i>fabG4</i> and <i>phaEC</i>	This study
pWLG5EC	Derivative of pL52, 13.5 kb, <i>fabG5</i> and <i>phaEC</i>	This study
pWLG6EC	Derivative of pL52, 13.4 kb, <i>fabG6</i> and <i>phaEC</i>	This study
pUBP	6.6-kb derivative of pUBP2 obtained by removing the pHH9 <i>ori</i> region	6
pUBPSG1	7.1-kb integration vector for disruption of <i>fabG1</i>	This study
pUBPSG2	7.0-kb integration vector for disruption of <i>fabG2</i>	This study
pUBPSG3	7.0-kb integration vector for disruption of <i>fabG3</i>	This study
pUBPSG4	7.0-kb integration vector for disruption of <i>fabG4</i>	This study
pUBPSG5	7.0-kb integration vector for disruption of <i>fabG5</i>	This study
pUBPSG6	7.0-kb integration vector for disruption of <i>fabG6</i>	This study
pUBPDG1	7.5-kb integration vector for knockout of <i>fabG1</i>	This study

acetoacetyl-CoA reductase in *H. hispanica*. It is responsible for providing (R)-3-HB-CoA for PHA biosynthesis in *Haloarcula* species, and interestingly, this enzyme also functions well in *Haloferax volcanii*, endowing this PHA-defective strain with the ability to accumulate PHA when cotransformed with PHA synthase genes.

#### MATERIALS AND METHODS

**Strains, plasmids, primers, and media.** The strains and plasmids used in this study are listed in Table 1. The primers are listed in Table 2. Plasmids used for gene disruption/knockout and expression were derived from pUBP (6) and pWL102 (13) (Table 1), respectively. These plasmids were usually constructed in *E. coli* JM109 and then introduced into *H. hispanica* or *H. volcanii* (39) by a polyethylene glycol-mediated transformation method (3).

*E. coli* JM109 was grown in Luria-Bertani (LB) medium at 37°C (33). When needed, ampicillin was added to a final concentration of 100 mg/liter. Generally, *H. hispanica* and *H. volcanii* strains were cultivated at 37°C in a nutrient-rich medium (AS-168) (6). For PHA accumulation analysis, *H. hispanica* and *H. volcanii* cells were first cultured at 37°C for 48 h in AS-168 medium, and then 5% (vol/vol) inocula were transferred to 100 ml MG medium (10 g/liter glucose in minimal medium) in shaking flasks (6) and cultivated for an additional 96 h. The pH was maintained manually at about 7.2 in MG medium. When required, mevinolin was added to a final concentration of 5 mg/liter for *H. volcanii* and *H. hispanica* transformants.

**RT-PCR.** Total RNA of *H. hispanica* AS2049 was extracted with Trizol reagent (Gibco BRL) from cells grown in MG medium for 72 h. Six primer pairs (*fabG1*RTF-*fabG1*RTR, *fabG2*RTF-*fabG2*RTR, *fabG3*RTF-*fabG3*RTR, *fabG4*RTF-*fabG4*RTR, *fabG5*RTF-*fabG5*RTR, and *fabG6*RTF-*fabG6*RTR) were designed specifically for the *fabG1* to *fabG6* genes, respectively (Table 2). The RNA samples were treated with RNase-free RQ1 DNase (Promega) to eliminate any DNA contamination, which was confirmed by a control PCR without prior reverse transcription (RT). The DNA-free RNA samples were then used as templates for RT-PCR with a OneStep RT-PCR kit (Qiagen) according to the manufacturer's instructions.

**Disruption of *fabG* genes via single crossover.** To distinguish the functions of the *FabG* paralogs in *H. hispanica* during PHA synthesis, the six *fabG* genes were disrupted by a single-crossover method. Briefly, a 475-bp DNA fragment located

in the middle of the *fabG1* gene was amplified with primer pair *fabG1*SF-*fabG1*SR (Table 2). The PCR product was sequenced and inserted into the suicide plasmid pUBP (Table 1). The resulting plasmid, pUBPSG1, was introduced into *H. hispanica* to disrupt the *fabG1* gene by single-crossover homologous recombination. The resulting mutant strain was named *H. hispanica* *fabG1*-1. Similarly, the other five *fabG*-disrupted mutants, named *H. hispanica* *fabG2*-1 to *fabG6*-1, were constructed as described above, using primer pairs *fabG2*SF-*fabG2*SR, *fabG3*SF-*fabG3*SR, *fabG4*SF-*fabG4*SR, *fabG5*SF-*fabG5*SR, and *fabG6*SF-*fabG6*SR (Table 2), respectively.

**Knockout and complementation of the *fabG1* gene.** To construct an in-frame *fabG1* deletion mutant, a 465-bp DNA fragment spanning the 5' region upstream of the *fabG1* gene and a 467-bp fragment spanning the 3' region downstream of the *fabG1* gene were amplified by use of primer pairs *fabG1*F2-*fabG1*R2 and *fabG1*F3-*fabG1*R3 (Table 2), respectively. These two PCR products were sequenced and cloned into the plasmid pUBP (Table 1). The resulting plasmid, pUBPDG1 (Table 1), was then introduced into *H. hispanica* to knock out the *fabG1* gene by double-crossover homologous recombination, generating a *fabG1*-deleted strain named *H. hispanica*  $\Delta$ *fabG1*. Primers *fabG1*F2 and *fabG1*R3 were used for identification of the single-crossover and double-crossover mutants by PCR analysis. To construct the complementation strain of *H. hispanica*  $\Delta$ *fabG1*, the *fabG1* gene was amplified with primers *fabG1*F1 and *fabG1*R1 and cloned into pL52 under the control of the *hsp5* promoter from *Halobacterium* (18). The resulting plasmid, pWLG1, was confirmed by sequencing and introduced into *H. hispanica*  $\Delta$ *fabG1* to check if the capability of PHA accumulation was restored.

**Coexpression of *fabG* genes with PHA synthase genes in *H. volcanii*.** For coexpression of *fabG1* with PHA synthase genes, the *phaEC* genes of *H. hispanica*, including the native promoter, were amplified with primers *phaEC*F and *phaEC*R and inserted into plasmid pWLG1, resulting in plasmid pWLG1EC. For the *fabG2* to *fabG6* genes, the respective coding sequences were amplified from *H. hispanica* by PCR with primer pairs *fabG2*F1-*fabG2*R1, *fabG3*F1-*fabG3*R1, *fabG4*F1-*fabG4*R1, *fabG5*F1-*fabG5*R1, and *fabG6*F1-*fabG6*R1, respectively, and cloned into pL52, resulting in pWLG2 to pWLG6, respectively. Next, the *phaEC* genes were cloned into pWLG2 to pWLG6, generating pWLG2EC to pWLG6EC, respectively, as described for pWLG1EC. In addition, the same DNA fragment of *phaEC* was inserted into pWL102 to construct plasmid pWLEC, which was used as a control plasmid in testing of the functions of the *fabG* genes. All of these plasmids were confirmed by DNA sequencing,

TABLE 2. Primers used in this study

Primer	Sequence (5'-3') <sup>a</sup>
fabG1RTF	GGGTCCTCACGCGGTATC
fabG1RTR	ACGCCGCCAGGTTGACG
fabG2RTF	TTCCGCTGTACGACTCTC
fabG2RTR	GCGGGAGGGCGTACTTCG
fabG3RTF	GACGGAGAGACAGCTATC
fabG3RTR	CCCGCCGCCATGACTCGA
fabG4RTF	AGGGTCAGCGGGCGATTA
fabG4RTR	CGTGAGGTTGATGTCGA
fabG5RTF	CGCGCACTTACGAGGACG
fabG5RTR	GGCCAGCAGTCCACTTTT
fabG6RTF	CGGTCTCACGGGCGGGCG
fabG6RTR	GCGCCGAAGAATACACCC
fabG1F1	TGCGACATATGAACCTGGATAATC
fabG1R1	TACTCTAGACTACCACTCCATCCGCC
fabG2F1	TGCGACATATGGACGTTCCGCTGT
fabG2R1	TACTCTAGACTACCACTCGATTCCGGC
fabG3F1	TGCGACATATGAGTGTAGTTGACA
fabG3R1	TACTCTAGATCACCGCGAGGTGTAGCC
fabG4F1	TGCGACATATGCCGAGATGTCAG
fabG4R1	TACTCTAGACTACCACTCGGGCTCTC
fabG5F1	TGCGACATATGGGTGTGCGGTGTCA
fabG5R1	TACTCTAGATCAATTGGTTGTGCGGCTG
fabG6F1	TGCGACATATGACAACACTCTCG
fabG6R1	TACTCTAGACTCAGGTCTCGTCACTCC
fabG1SF	TTATAAGCTTAGCCCGTCCGTCGTCGA
fabG1SR	ATTGGTACCACGCGTTCGGGCACCTC
fabG2SF	TTATAAGCTTGAAGGCAATCGCCGACGG
fabG2SR	ATTGGTACCGTCCGCCCCGACTGGCTC
fabG3SF	TTATAAGCTTTGGCTATCGCGGACGTGA
fabG3SR	ATTGGTACCGCCTGTTTCTGCGGGACG
fabG4SF	TTATAAGCTTGGCAGCAGGACAACGTCG
fabG4SR	ATTGGTACCACATGTACGGCGCGCCCTG
fabG5SF	TTATAAGCTTAGATCGCGGCCGTTCCG
fabG5SR	ATTGGTACCGCGTTCGACCGAGCCCAT
fabG6SF	TTATAAGCTTCCATTTGCAAGACGTACG
fabG6SR	ATTGGTACCATGCCGCGGAGCTGGAC
phaECF	ATTGGTACCAGCTCGAAGAAGTGCAG
phaECR	TATCCATGGCTCGCACCCCGGCAGACT
fabG1F2	ATAAAGCTTTCGGCACCATCACGCAGA
fabG1R2	ATACTGCAGCGCCCGGACTTTCATCGAG
fabG1F3	TTAGGATCCGGGATGGAGTGGTGGAT
fabG1R3	ATAGGTACCCAACGCCTTCTGAATGC

<sup>a</sup> Sequences representing restriction sites are shown in bold.

and the *H. volcanii* cells harboring these plasmids were subjected to analysis of PHA accumulation.

**Analysis of PHA.** The PHA contents and compositions in dry cells were determined by gas chromatography (GC) with an Agilent GC-6820 instrument as described previously (6, 19). Benzoic acid was used as an internal standard to calculate the amount of PHA.

**Protein preparation and acetoacetyl-CoA reductase assay.** For enzyme activity assay, *H. hispanica* cells cultivated in AS-168 medium for 72 h were harvested by centrifugation and then suspended with 3.4 M KCl in 20 mM Tris-HCl (pH 7.5). 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 concentration of cellular proteins was determined with a bicinchoninic acid protein assay kit (Pierce).

The activity of acetoacetyl-CoA reductase was measured spectrophotometrically at 340 nm by recording the oxidation of NADPH or NADH as described by Senior and Dawes (34), with some minor modifications. Briefly, assays were carried out at 25°C in a final volume of 200 μl containing 20 mM Tris-HCl (pH 7.5), 3.4 M KCl, 20 μM acetoacetyl-CoA (Sigma), 100 μM NADPH or NADH (Roche), and 50 μg of protein extracts. Negative controls in which acetoacetyl-CoA was omitted from the reaction mixture were performed to subtract the activities of any possible NAD(P)H oxidases in the protein extracts. The reaction was initiated by the addition of crude extract samples at 25°C. A decrease in the absorbance at 340 nm was recorded for 10 min. The initial rate was used to

calculate the enzymatic activity. The oxidation of 1 μmol NADPH/NADH per minute corresponded to an enzyme activity of 1 U.

## RESULTS

**Genome-wide screening of reductase genes for supplying PHA precursors in *H. hispanica*.** Both *H. marismortui* and *H. hispanica* are capable of synthesizing SCL-PHAs, and the PHA synthase genes in these two haloarchaeal strains were characterized recently (6). However, the PHA-specific acetoacetyl-CoA reductase (PhaB), which provides the (*R*)-3-HB-CoA precursor for biosynthesis of either PHB or PHBV in bacteria, has not yet been identified in these haloarchaea. Interestingly, multiple FabG paralogs are present in *H. marismortui* ATCC 43049 (1), and at least six homologous proteins, FabG1 to FabG6, named like their counterparts in *H. marismortui*, are also observed in the *H. hispanica* genome, with identities ranging from 95.0% to 97.6% for each pair of counterparts.

Like the case for PhaB (previously named FabG) in *Synechocystis* sp. strain PCC6803 (36), the catalytically active triad (Ser, Tyr, and Lys) and the N-terminal cofactor binding sequence (Gly motif [GlyXXXGlyXGly]) of SDRs (23) are present in all of the FabG paralogs from *H. hispanica* (Fig. 1A; see Fig. S1 in the supplemental material). Moreover, the proposed signature sequence of the Gly motif of PHA-specific acetoacetyl-CoA reductases (ValThrGlyXXXGlyIleGly) (36) is completely conserved in FabG1, FabG2, and FabG5 (Fig. 1A).

To understand the phylogenetic relationships between these haloarchaeal FabG paralogs and well-known bacterial FabGs or PhaBs, a phylogenetic tree was constructed (Fig. 1B). To our surprise, while the confirmed bacterial PhaBs (GenBank accession no. NP\_441203.1, P45375.1, and YP\_725942.1) (15, 28, 36), FabGs (NP\_215999 and AAK04872.1) (24, 38), and those SDRs with both PhaB and FabG activities (AAA23739.1 and BAE19680.1) (21, 22, 35) were clustered in several specific subgroups, the haloarchaeal FabG1 to FabG6 proteins were highly divergent from their bacterial counterparts (Fig. 1B). The levels of amino acid identity between PhaBs and the six haloarchaeal FabG paralogs ranged from 25% to 38%, with FabG1 exhibiting the highest level of homology. However, even FabG1 was only distantly related to the bacterial PhaBs (Fig. 1B), and hence the involvement of FabG1 and other FabG paralogs in supplying (*R*)-3-HB-CoA precursors for PHA biosynthesis in *Haloarcula* species remained to be clarified experimentally.

**Analysis of the involvement of *fabG* genes in PHA synthesis in *H. hispanica*.** Prior to exploring the possible involvements of the FabG paralogs during PHA synthesis in *H. hispanica*, RT-PCR using gene-specific primers was first performed to check whether they were transcribed under PHA-accumulating conditions (Table 2). The results demonstrated that all six genes were actively transcribed, but the transcription levels were relatively higher for the *fabG1*, *fabG3*, and *fabG4* genes (Fig. 2). Hence, all of the FabG paralogs might play roles in metabolism of this archaeon.

In order to identify which FabG paralog might be involved in PHA synthesis, the *fabG* genes were then disrupted by a single-crossover recombination method (see Materials and Methods) (Fig. 3A). The successful gene disruptions for *fabG1* to *fabG6* were confirmed by PCR analysis (data not shown). The

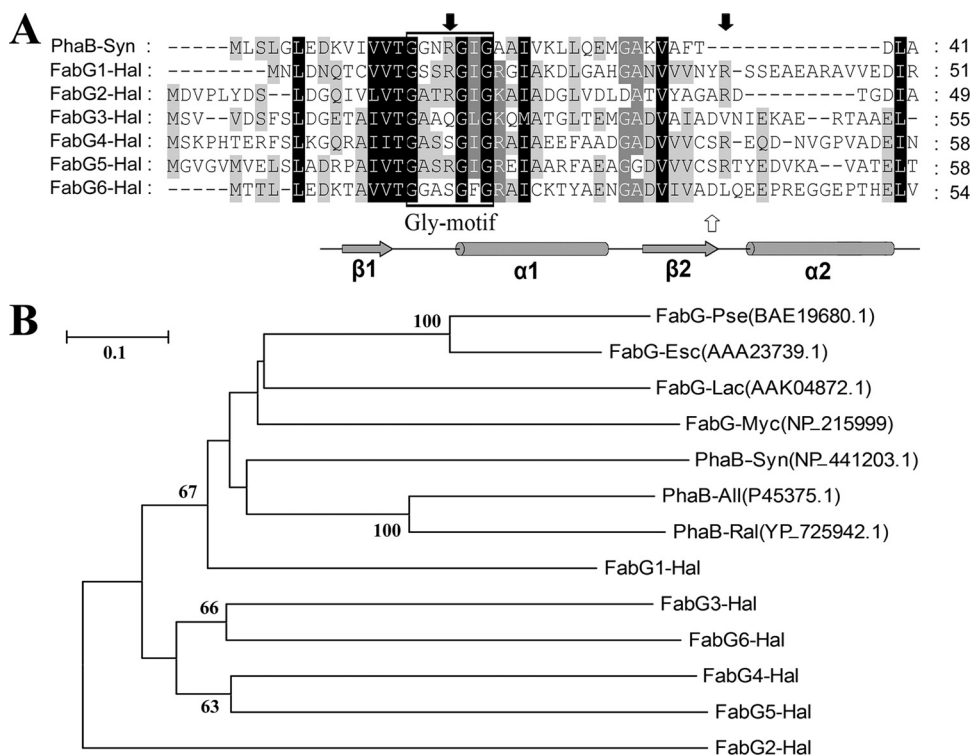


FIG. 1. (A) Multiple alignments of N termini of PhaB from *Synechococcus* sp. strain MA19 (Syn) and six FabG (FabG1 to FabG6) proteins from *H. hispanica* AS2049 (Hal). The Gly motif is boxed. The partial secondary structure elements of the sequences are shown below the alignments. The symbols “ $\alpha$ ” and “ $\beta$ ” represent  $\alpha$ -helices and  $\beta$ -strands, respectively. The black and white vertical arrows point to the key basic and acidic residues, respectively, determining the type of cofactor, i.e., NADPH or NADH (11). Black shading indicates identical residues; gray shading indicates similar residues. (B) Phylogenetic analysis of FabGs and PhaBs from *H. hispanica* and bacteria. The phylogenetic tree was constructed by using the neighbor-joining method. Bootstrap values of  $>50\%$  (1,000 replicates) are shown at the nodes. GenBank accession numbers for bacterial proteins are shown in parentheses. Full-length sequences of the FabG paralogs from *H. hispanica* and their alignment with bacterial PhaBs are shown in Fig. S1 in the supplemental material.

resultant six *fabG*-interrupted mutants, named *H. hispanica* fabG1-1 to fabG6-1, were subjected to the following phenotypic analyses.

Interestingly, all of the *fabG* mutants showed no significant difference in growth compared with the wild-type strain, as evaluated by their cell dry weight (CDW) (Table 3). Moreover, disruption of the *fabG2* to *fabG6* genes had no evident effects on PHA accumulation, including both the PHA content and monomer ratios (Table 3). Significantly, though, GC analysis revealed that PHA synthesis was completely abolished in *H.*

*hispanica* fabG1-1 cells. These results inferred that FabG1, but not the other FabG paralogs, might be the PHA precursor supplying enzyme and might play an important role in PHA biosynthesis.

**FabG1 is indispensable for PHA synthesis in *H. hispanica*.** Analysis of the gene location of *fabG1* in the chromosome of *H. hispanica* revealed two downstream genes encoding an acetyl-CoA carboxylase  $\alpha$  subunit (AccA) and a conserved hypothetical protein. There was only a 1-base spacing between any two adjacent genes, suggesting that these three genes might be cotranscribed. Thus, the large insertion in the *fabG1* gene in the *fabG1*-disrupted mutant might generate polar effects on downstream genes, which might also influence the metabolism of PHA synthesis. Therefore, gene knockout and complementation analysis was carried out to further clarify the function of FabG1 during PHA biosynthesis in *H. hispanica*.

With a double-crossover homologous recombination strategy (Fig. 3B), the *fabG1* gene was deleted in frame, resulting in a mutant strain named *H. hispanica*  $\Delta$ *fabG1*. The genotype of the  $\Delta$ *fabG1* strain was confirmed by PCR analysis (Fig. 3C). Significantly, the  $\Delta$ *fabG1* cells lost the ability to accumulate PHBV, as demonstrated by GC analysis (Fig. 4B). In addition, when the  $\Delta$ *fabG1* strain was transformed with the *fabG1* expression plasmid pWLG1, the capability of PHA accumulation was restored (Fig. 4C). These results further demonstrated

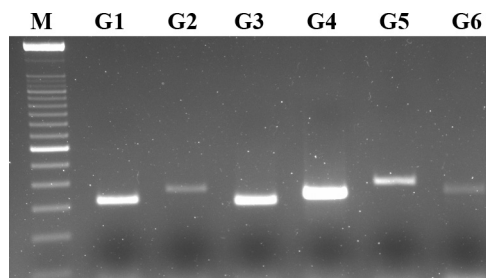


FIG. 2. RT-PCR analysis of six *fabG* genes under PHA-accumulating conditions. Lane G1, *fabG1* (320 bp); lane G2, *fabG2* (366 bp); lane G3, *fabG3* (313 bp); lane G4, *fabG4* (342 bp); lane G5, *fabG5* (389 bp); lane G6, *fabG6* (349 bp); lane M, 100-bp DNA ladder.

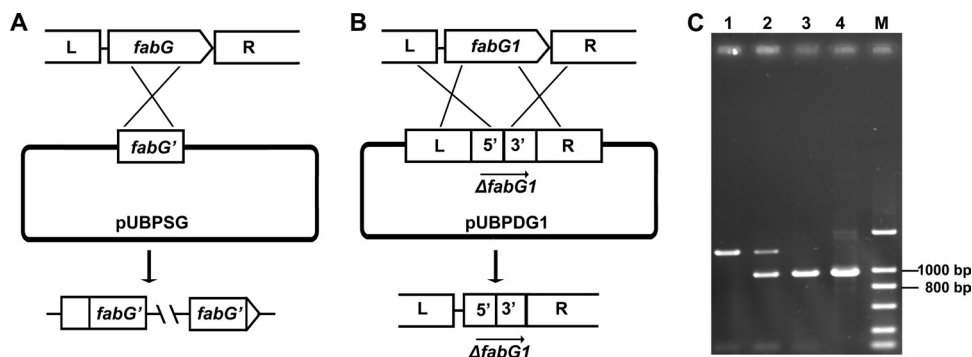


FIG. 3. Disruption of six *fabG* genes and knockout of *fabG1* gene in *H. hispanica*. (A) Schematic representation of disruption of six *fabG* genes in *H. hispanica* via a single-crossover strategy. Plasmid pUBPSG represents plasmids pUBPSG1 to pUBPSG6. (B) Schematic representation of *fabG1* gene knockout in *H. hispanica* via a double-crossover strategy with plasmid pUBPDG1. (C) PCR identification of *fabG1*-deleted  $\Delta fabG1$  strain, using primers *fabG1F2* and *fabG1R3*. Lane 1, Genomic DNA from wild-type strain, used as a negative control (1,445 bp); lane 2, genomic DNA from single-crossover strain integrated by pUBPDG1 (932 bp and 1,445 bp); lane 3, genomic DNA from double-crossover  $\Delta fabG1$  strain (932 bp); lane 4, plasmid pUBPDG1, used as a positive control.

that the *fabG1* gene was indeed indispensable for PHA synthesis and was the PHA precursor-supplying enzyme in *H. hispanica*.

**Establishment of PHA biosynthesis pathway in *H. volcanii*.** *H. volcanii* DS70 is a model haloarchaeon which is easy to use for genetic manipulation but defective in PHA biosynthesis. Therefore, it is a suitable host for verification of PHA biosynthesis genes from other haloarchaea. BLAT analysis revealed that there was neither a PhaB nor PHA synthase homolog in the genome of *H. volcanii* (The UCSC Archaeal Genome Browser [http://archaea.ucsc.edu/]). Therefore, to verify the function of FabGs in *H. volcanii*, it is necessary to heterologously express the *fabG* gene with PHA synthase genes (*phaEC*). These kinds of expression plasmids, pWLG1EC to pWLG6EC (Table 1), were introduced into the *H. volcanii* DS70 cells, with pWLEC harboring *phaEC* genes as the control. Table 3 shows the PHA accumulation in *H. volcanii* DS70 and its transformants. Notably, neither the wild-type strain nor the recombinant harboring plasmid pWLEC accumulated detectable PHA, confirming that *H. volcanii* DS70 was deficient not only in polymerization enzymes but also in PHA precursor-

supplying enzymes. The seven recombinant strains harboring the heterologous *fabG* plus *phaEC* genes showed no significant difference in cell growth compared with the wild-type strain of *H. volcanii* (Table 3). Significantly, only the coexpression of *fabG1* and *phaEC* in DS70 resulted in detectable PHA accumulation, with ~3% (wt/wt) CDW (Table 3). In contrast, the other five FabGs (FabG2 to FabG6) likely had no roles in supplying PHA precursor, as no PHA accumulation was detected after their expression in *H. volcanii*. These results demonstrated that FabG1 from *H. hispanica*, but not the other five FabG paralogs, could supply precursors for SCL-PHA synthesis in both *Haloarcula* and *Haloferax* species.

**FabG1 exhibits NADPH-dependent activity of acetoacetyl-CoA reductase.** To biochemically confirm the function of FabG1, an enzyme activity assay was established to investigate whether it could convert acetoacetyl-CoA into (*R*)-3-HB-CoA, the main precursor for SCL-PHAs. Since the *fabG* genes were constitutively expressed under both nutrient-rich (AS-168 medium) and nutrient-limited (MG medium) conditions (data not shown), the cell crude extracts of *H. hispanica* strains cultivated in AS-168 medium were subjected to the acetoacetyl-

TABLE 3. PHA accumulation in *H. volcanii* and *H. hispanica* strains<sup>a</sup>

Strain	CDW (g/liter)	PHA content (% [wt/wt])	PHA concn (g/liter)	PHA composition (mol%)	
				3-HB (C <sub>4</sub> )	3-HV (C <sub>5</sub> )
<i>H. hispanica</i> AS2049	10.2 ± 0.4	8.6 ± 0.6	0.9 ± 0.1	96.6 ± 0.4	3.4 ± 0.4
<i>H. hispanica</i> fabG1-1	10.4 ± 0.2	ND	ND	ND	ND
<i>H. hispanica</i> fabG2-1	11.2 ± 0.5	7.3 ± 0.4	0.8 ± 0.1	96.8 ± 0.5	3.2 ± 0.5
<i>H. hispanica</i> fabG3-1	10.7 ± 0.3	6.9 ± 1.5	0.7 ± 0.2	96.3 ± 0.2	3.7 ± 0.2
<i>H. hispanica</i> fabG4-1	11.0 ± 0.5	7.5 ± 0.8	0.8 ± 0.1	96.1 ± 0.3	3.9 ± 0.3
<i>H. hispanica</i> fabG5-1	10.5 ± 0.2	7.7 ± 0.3	0.8 ± 0.1	96.7 ± 0.4	3.3 ± 0.4
<i>H. hispanica</i> fabG6-1	10.6 ± 0.1	7.5 ± 0.8	0.8 ± 0.1	96.6 ± 0.2	3.4 ± 0.2
<i>H. volcanii</i> DS70	4.8 ± 0.0	ND	ND	ND	ND
<i>H. volcanii</i> (pWLEC)	5.7 ± 0.5	ND	ND	ND	ND
<i>H. volcanii</i> (pWLG1EC)	4.2 ± 0.5	3.1 ± 0.4	0.5 ± 0.0	98.5 ± 0.2	1.5 ± 0.2
<i>H. volcanii</i> (pWLG2EC)	5.5 ± 0.4	ND	ND	ND	ND
<i>H. volcanii</i> (pWLG3EC)	4.6 ± 0.1	ND	ND	ND	ND
<i>H. volcanii</i> (pWLG4EC)	5.1 ± 0.6	ND	ND	ND	ND
<i>H. volcanii</i> (pWLG5EC)	5.0 ± 0.5	ND	ND	ND	ND
<i>H. volcanii</i> (pWLG6EC)	4.9 ± 0.4	ND	ND	ND	ND

<sup>a</sup> The cells were cultured in MG medium at 37°C for 96 h. Data shown are means ± standard deviations (*n* = 3). ND, not detectable.

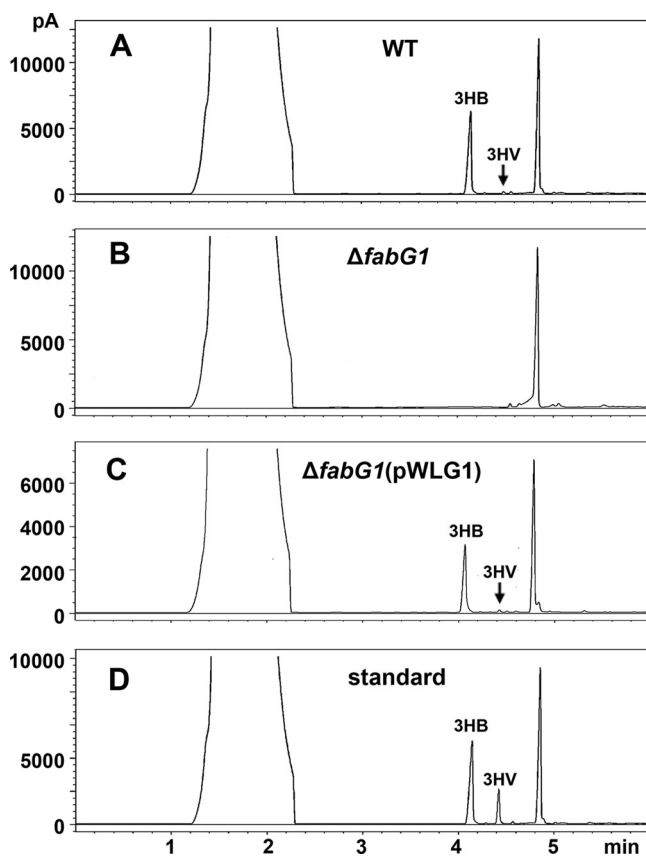


FIG. 4. GC analysis of PHBV accumulation in *H. hispanica* and recombinant strains. (A) *H. hispanica* wild-type strain; (B)  $\Delta fabG1$  strain; (C)  $\Delta fabG1$  strain harboring plasmid pWLG1; (D) PHBV standard (Sigma). The peaks marked 3HB and 3HV represent 3-HB methyl ester and 3-HV methyl ester, respectively. The peak at 4.85 min represents the methyl ester product of an internal standard (1 ng of benzoic acid). The *H. hispanica* recombinants were cultivated in MG medium at 37°C for 96 h. The 3-HB and 3-HV monomers of PHA synthesized by wild-type *H. hispanica* were confirmed by 400-MHz  $^1H$  nuclear magnetic resonance analysis (data not shown).

CoA reductase activity assay, supplying NADPH or NADH as the cofactor (Table 4). Significantly, when the *fabG1* gene was disrupted or knocked out, the NADPH-dependent activity of acetoacetyl-CoA reductase in the mutant, the  $\Delta fabG1$  or *fabG1-1* strain, was greatly decreased, from ~20 to ~0.5 U/g crude extract proteins (Table 4). Correspondingly, when the *fabG1* gene was reexpressed in the  $\Delta fabG1$  strain, the NADPH-dependent acetoacetyl-CoA reductase activity (25.6 U/g) (Table 4) was restored. Under both conditions, however, the NADH-dependent acetoacetyl-CoA reductase activities were not significantly affected. These results indicate that FabG1 is indeed a NADPH-dependent acetoacetyl-CoA reductase.

To our surprise, when the other five *fabG* paralogs (*fabG2* to *fabG6*) were disrupted, neither the NADPH- nor NADH-dependent acetoacetyl-CoA reductase activity was significantly changed (Table 4). It is possible that these FabG paralogs prefer their natural substrates (i.e., 3-ketoacyl-ACP); hence, little acetoacetyl-CoA reductase activity would be present. It is also possible that these FabG paralogs evolved to serve

other, unknown functions, which remain to be investigated in the future. In any case, our results incontrovertibly demonstrate that FabG1 is distinct from its five paralogs and evolved to be a PHA-specific acetoacetyl-CoA reductase, using NADPH as a cofactor, in the *Haloarcula* genus.

## DISCUSSION

FabG and PhaB are members of a vast protein family, the SDR superfamily, which catalyze a wide variety of NAD(P)(H)-dependent oxidation/reduction reactions (23). Annotation of *fabG*- or *phaB*-homologous genes is particularly problematic (2, 17, 36). In the present study, we combined bioinformatics, classic genetic techniques, and biochemical methods to elucidate the functions of several *fabG*-paralogous genes in PHA biosynthesis in *H. hispanica*. We demonstrated that the *fabG1* gene actually encodes a NADPH-dependent acetoacetyl-CoA reductase (PhaB) which is indispensable for converting acetoacetyl-CoA into (*R*)-3-HB-CoA for PHBV biosynthesis in this haloarchaeon. To our knowledge, this is the first PHA precursor-supplying gene experimentally identified so far in the domain of *Archaea*.

There are multiple FabG paralogs in the sequenced genomes of several haloarchaeal species, including *H. marismortui* (1), *H. hispanica* (our unpublished genome data), and *H. volcanii* (The UCSC Archaeal Genome Browser [http://archaea.ucsc.edu/]). Our results indicate that FabG1 in *H. hispanica*, and possibly its counterpart (with 97% identity) in *H. marismortui*, has evolved certain distinct functions in PHA biosynthesis. Biochemical assay revealed that only FabG1 among the six paralogs is a NADPH-dependent acetoacetyl-CoA reductase, and genetic and metabolic evidence supports the observation that only FabG1 could convert acetoacetyl-CoA into (*R*)-3-HB-CoA and provide this precursor for PHA synthesis. While the exact functions of FabG2 to FabG6 remain unclear, the NADH-dependent reductase activity (<3 U/g crude extract proteins) detected in all of the *fabG* mutants infers that there might be other NADH-dependent acetoacetyl-CoA reductases. These NADH-dependent enzymes, if any, might just convert acetoacetyl-CoA into (*S*)-3-HB-CoA (7), which is unable to be incorporated into PHBV by PHA synthases. Interestingly, BLAT analysis of the genome of *H. volcanii* DS2 revealed that there were no homol-

TABLE 4. Activity assay of acetoacetyl-CoA reductase in *H. hispanica* strains

<i>H. hispanica</i> strain	Acetoacetyl-CoA reductase activity (U/g crude extract proteins) <sup>a</sup>	
	NADPH	NADH
Wild type	19.4	2.8
<i>fabG1-1</i> mutant	0.4	2.5
<i>fabG2-1</i> mutant	18.7	2.6
<i>fabG3-1</i> mutant	17.1	2.5
<i>fabG4-1</i> mutant	18.5	2.7
<i>fabG5-1</i> mutant	17.3	2.6
<i>fabG6-1</i> mutant	20.6	3.1
$\Delta fabG1$ mutant	0.5	2.6
$\Delta fabG1$ (pWLG1) mutant	25.6	3.0

<sup>a</sup> Enzyme solutions used in this assay were crude extracts of wild-type or recombinant strains as described in Materials and Methods. The experiment was repeated three times, and representative results are shown.

ogous genes encoding either PhaEC or FabG1, while those for the other five FabG paralogs were detected. This observation was consistent with our heterologous expression results showing that only the introduction of *fabG1* and *phaEC* could confer on the PHA-defective strain the ability to synthesize PHA. The low content of PHA in the recombinant *H. volcanii* strain was likely due to its poor capability of carbohydrate utilization (5).

Genome-wide analysis revealed that there are several  $\beta$ -ketoacyl thiolase genes in *H. marismortui* as well as in *H. hispanica*. In the present study, we showed that the FabG1 protein was responsible for providing 3-HB-CoA, and coexpression of the *fabG1* and *phaEC* genes was able to reestablish the PHA synthesis pathway in *H. volcanii*. These results suggested that  $\beta$ -ketoacyl thiolases do indeed exist in both *H. hispanica* and *H. volcanii*. In fact, we detected the activity of  $\beta$ -ketoacyl thiolase in the crude extracts of both strains (data not shown). At present, we are investigating which  $\beta$ -ketoacyl thiolase catalyzes acetoacetyl-CoA synthesis and hence is involved in PHA synthesis. Thus, the PHA biosynthesis pathway from acetyl-CoA, catalyzed by  $\beta$ -ketoacyl thiolase, acetoacetyl-CoA reductase, and PHA synthase, as distributed in *Bacteria*, likely also exists in the domain of *Archaea*. However, for PHB-accumulating haloarchaeal *Natrialba* strain 56, no enzyme activity of acetoacetyl-CoA reductase or  $\beta$ -ketoacyl thiolase was detected in the crude extract (8, 9), indicating that a different metabolic route toward PHB biosynthesis might be employed.

In bacteria, the PHA-specific acetoacetyl-CoA reductase, named PhaB, has two other conserved residues (Val and Ile) in the cofactor binding sequence (ValThrGlyXXXGlyIleGly). Similarly, FabG1 was also found to share these conserved amino acids. Most bacterial PhaB proteins use NADPH as the cofactor (7, 27), except that PhaB from *Allochrochromatium vinosum* (originally named *Chromatium vinosum*) has been reported to be NADH dependent (15). Likewise, the FabG1 protein was experimentally proven to be NADPH dependent and functioned as an (*R*)-3-HB-CoA-supplying enzyme. The cofactor of FabG1 could also be predicted by the method developed by Kallberg et al. (11). They proposed that if there is no acidic residue (Asp) at the end of the second  $\beta$ -strand and a basic residue (Arg or Lys) is located in the Gly motif or at the first loop position after the second  $\beta$ -strand, like in FabG1, NADPH will be used as the cofactor (Fig. 1A). Therefore, based on their conserved motif, function, and enzyme activity, we recommend renaming the FabG1 proteins of both *H. hispanica* and *H. marismortui* as PhaB proteins, the PHA-specific acetoacetyl-CoA reductases of halophilic archaea.

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