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Activity and Transcriptional Regulation of Bacterial Protein-Like Glycerol-3-Phosphate Dehydrogenase of the Haloarchaea in *Haloferax volcanii*▽†

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Glycerol is a primary energy source for heterotrophic haloarchaea and a major component of "salty" biodiesel waste. Glycerol is catabolized solely by glycerol kinase (encoded by *glpK*) to glycerol-3-phosphate (G3P) in *Haloferax volcanii*. Here we characterized the next critical step of this metabolic pathway: the conversion of G3P to dihydroxyacetone phosphate by G3P dehydrogenase (G3PDH). *H. volcanii* harbors two putative G3PDH operons: (i) *glpA1B1C1*, located on the chromosome within the neighborhood of *glpK*, and (ii) *glpA2B2C2*, on megaplasmid pHV4. Analysis of knockout strains revealed that *glpA1* (and not *glpA2*) is required for growth on glycerol. However, both *glpA1* and *glpA2* could complement a *glpA1* knockout strain (when expressed from a strong promoter in *trans*) and were required for the total G3PDH activity of cell lysates. The *glpA1B1C1*, *glpK*, *glpF* (encoding a putative glycerol facilitator), and *ptsH2* (encoding a homolog of the bacterial phosphotransferase system protein Hpr) genes were transcriptionally linked and appeared to be under the control of a strong, G3P-inducible promoter upstream of *glpA1*. Overall, this study provides fundamental insights into glycerol metabolism in *H. volcanii* and enhances our understanding of central metabolic pathways of haloarchaea.

Glycerol is a highly abundant energy source in hypersaline environments as a result of leakage from and lysis of *Dunaliella* cells, which are known to accumulate glycerol in molar quantities as an organic, osmotic solute (3, 5, 7, 32). Thus, glycerol is a primary energy source for heterotrophic members of this community.

In biological systems, glycerol is metabolized to dihydroxyacetone phosphate (DHAP) by one of two routes: (i) phosphorylation by glycerol kinase and subsequent conversion of *sn*-glycerol-3-phosphate (G3P) into DHAP through G3P dehydrogenase (G3PDH) or (ii) oxidation by glycerol dehydrogenase to form dihydroxyacetone (DHA), which is subsequently phosphorylated by an ATP-dependent or phosphoenolpyruvate:phosphotransferase system (PEP:PTS)-dependent DHA kinase to form DHAP. Once generated from glycerol, DHAP can be channeled into metabolic intermediates, including pyruvate, G3P, and/or *sn*-glycerol-1-phosphate (G1P).

Recently, we demonstrated through *Haloferax volcanii* that haloarchaea require glycerol kinase (encoded by *glpK*) for the catabolism of glycerol (27). These results suggest that (i) G3PDH is needed for glycerol metabolism and (ii) the homologs of bacterial PEP:PTS-dependent DHA kinase are not needed for glycerol catabolism in *H. volcanii* but may serve in the metabolism of DHA overflow products generated by other members of the hypersaline community, such as *Dunaliella salina* (4).

In this study, we investigated the oxidation of G3P to DHAP by G3PDH, a metabolic step likely to be central to glycerol catabolism and subsequent to the phosphorylation of glycerol by glycerol kinase in haloarchaea. Since archaea use G1PDH (encoded by *egsA*) to convert DHAP to G1P for the biosynthesis of phospholipids, G3PDH homologs are not common in this domain (19, 23). In contrast, bacteria and eukaryotes use G3PDH to synthesize G3P for the backbones of their membrane lipids. Although previous work has demonstrated an archaeal G3PDH, this enzyme (GspA) has an unusual preference for $NADP^+$ (26). Furthermore, GspA is not a bacterial protein-like G3PDH; instead, it is a close homolog of the products of open reading frames (ORFs) from only a few archaea (*Archaeoglobus fulgidus*, *Methanothermobacter thermoautotrophicus*, *Aeropyrum pernix*).

Here we provide evidence that bacterial protein-like G3PDH homologs are common among haloarchaea and are required for the catabolism of glycerol in *H. volcanii*. We also demonstrate that the central G3PDH activity of *H. volcanii* is encoded by a glycerol metabolic operon on the main chromosome that includes not only *glpA1B1C1* (encoding G3PDH complex I) but also the downstream *glpK* (encoding glycerol kinase), *glpF* (encoding a putative glycerol facilitator), and *ptsH2* (encoding a bacterial protein-like PTS Hpr homolog) genes. This glycerol metabolic operon is under transcriptional control from a strong G3P-inducible promoter ($P1_{glpA1}$) upstream of *glpA1* and possibly from downstream promoters ($P2_{glpK}$). A large transcript (spanning the entire operon) that may undergo nucleolytic cleavage into shorter transcripts of differential stability was detected. Our findings not only provide the first molecular characterization of a bacterial protein-like G3PDH complex in archaea but also shed light on the central pathway of glycerol metabolism in haloarchaea.

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MATERIALS AND METHODS

Materials. Biochemicals used for analysis of G3PDH activity were purchased from Sigma-Aldrich (St. Louis, MO). Other organic and inorganic molecular biology-grade chemicals were from Fisher Scientific (Atlanta, GA) and Bio-Rad (Hercules, CA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IA). DNA polymerases and modifying enzymes were from New England Biolabs (Ipswich, MA).

Strains, media, and plasmids. Strains, oligonucleotide primers, and plasmids are summarized in Tables S1 and S2 in the supplemental material. *Escherichia coli* TOP10 was used for routine recombinant DNA experiments. *H. volcanii* strains were transformed (8) with plasmid DNA isolated from *E. coli* GM2163 by use of the QIAprep spin miniprep kit (Qiagen, Valencia, CA). *E. coli* strains were grown at 37°C (200 rpm) in Luria-Bertani medium supplemented with 100 mg per liter ampicillin as needed. *H. volcanii* strains were grown at 42°C (200 rpm) in Casamino Acids (CA) and minimal medium (MM) with formulae according to *The Halohandbook* (12) except that MM was supplemented with glycerol (Gly MM), glucose (Glu MM), and/or succinate (Suc MM) at 20 mM each, unless otherwise stated. Novobiocin (0.1 $\mu g \cdot ml^{-1}$), 5-fluoroorotic acid (5-FOA) (50 $\mu g \cdot ml^{-1}$), and uracil (10 and 50 $\mu g \cdot ml^{-1}$ for growth in the presence and absence of 5-FOA, respectively) were included as needed, and tryptophan (Trp) (820 $\mu g \cdot ml^{-1}$) was added where indicated. Uracil and 5-FOA were solubilized in 100% (vol/vol) dimethyl sulfoxide (DMSO) at 50 $mg \cdot ml^{-1}$ prior to addition to the growth medium. For anaerobic growth on glycerol, *H. volcanii* strains were grown twice aerobically on YPC medium (12) to log phase (2 ml in 13- by 100-mm tubes; 200 rpm) and were inoculated at 1% (vol/vol) for anaerobic growth in 10-ml screw-cap tubes on YPC medium supplemented with 100 mM DMSO with or without 20 mM glycerol (with growth dependent on the presence of glycerol).

For enzyme activity and RNA extraction experiments, *H. volcanii* cells were freshly inoculated from −80°C glycerol stocks onto agar-based media. Cells were subcultured twice during log phase and were used as an inoculum for final analysis under various conditions. Each subculture was inoculated to a final optical density at 600 nm (OD_{600}) of 0.03 to 0.04. For G3PDH enzyme activity assays and RNA preparation, cells were grown in 25 ml of medium in 250-ml flasks. For β-galactosidase activity measurements, cells were grown in 3 ml of medium in 13- by 100-cm culture tubes. Cell growth was monitored by an increase in OD_{600}.

Chromosomal knockout of *glpA1* and *glpA2*. Chromosomal *glpA1* (HVO_1538) and pHV4-based *glpA2* (HVO_A0269), encoding homologs of G3PDH subunit A, were deleted from the genome of *H. volcanii* H26 (Δ*pyrE2*) by using the markerless *pyrE2*-based pop-in/pop-out method (1, 6) and DS2 genome sequence information (16). Details on the single and double knockouts of *glpA1* and *glpA2* are outlined in Tables S1 and S2 in the supplemental material.

G3PDH activity assays. Log-phase cells (OD_{600}, 0.3 to 0.5) were harvested by centrifugation (20 min, 4,300 × *g*, 4°C), washed once with 20 ml buffer A (100 mM Tris-HCl at pH 7.5, 2 M NaCl), resuspended in 1 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by sonication (4 times, for 20 s each time, at 140 W). Debris was removed by centrifugation (10 min, 12,000 × *g*, 4°C). The protein concentration was estimated using the Bradford assay with bovine serum albumin as a standard. Enzyme activities were carried out aerobically at 42°C in a 96-well microtiter plate. Reaction mixtures (0.1 ml) contained 5 mM DHAP, 0.25 mM NADH, 2 M NaCl, and 1 to 4 μg cell lysate in buffer A. The change in absorbance at 340 nm (A_{340}) for reaction mixtures containing no substrate (DHAP) was subtracted from that for reaction mixtures in which the substrate was included to yield the overall change in absorbance. Reaction mixtures containing boiled enzyme and no NADH were included as negative controls. One unit of enzyme activity is defined as 1 μmol substrate consumed or product formed per min with a molar extinction coefficient of 6,220 $M^{-1} \cdot cm^{-1}$ at 340 nm.

RNA purification. For reverse transcription-PCR (RT-PCR), total RNA was isolated from *H. volcanii* H26 grown to log phase on Gly MM using RNeasy RNA purification columns (Qiagen). RNA was treated with amplification-grade DNase I according to the supplier's recommendations (Sigma-Aldrich), with the following modification: RNA was incubated with 3 U enzyme per μg RNA for 45 min at room temperature. Total RNA used for Northern blot analysis was prepared from *H. volcanii* strain H26 (grown to log phase on Glu MM or Gly Glu MM) using TRI reagent (Sigma), followed by DNase I treatment (as described above). The integrity of RNA was determined by agarose gel electrophoresis, and the RNA concentration was determined by A_{260} using a SmartSpec 3000 spectrophotometer (Bio-Rad).

RT-PCR analysis. Total RNA (0.1 μg) was reverse transcribed into cDNA using an iScript kit (Bio-Rad) (25°C for 5 min, 42°C for 30 min, 85°C for 5 min). Specific cDNAs were amplified by PCR using the primers listed in Table S2 in the supplemental material, *Taq* DNA polymerase with an appropriate buffer, a solution of mixed deoxynucleotides, and an iCycler (Bio-Rad). Reaction mixtures were preheated to 95°C (4 min), followed by 35 amplification cycles consisting of denaturation (30 s at 95°C), annealing (1 min at the temperatures listed in Table S2 in the supplemental material), and elongation (41 s at 72°C), after which a final extension was performed at 72°C (10 min). For each RT-PCR, controls were included to exclude genomic DNA contamination, and RT-PCR products were sequenced to confirm primer pair specificity.

Northern blot analysis. Total RNA (10 μg per lane) was denatured and fractionated by electrophoresis (4 h, 50 V) using formaldehyde–0.8% (wt/vol) agarose gels in 1× morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS [pH 7.0], 5 mM sodium acetate, 1 mM EDTA) according to standard procedures (2). RNA molecular mass standards labeled with 2′ dUTP coupled by an 11-atom spacer to digoxigenin (DIG-11-dUTP) (0.3- to 6.9-kb RNA ladder; Roche Molecular Biochemicals, Indianapolis, IN) were included. After several rinses with deionized water, the gel was incubated (45 min) in 10× saline sodium citrate (SSC) (where 20× SSC is 3 M NaCl plus 0.3 M sodium citrate [pH 7.0]). RNA was transferred to a BrightStar-Plus nylon membrane (Ambion, Austin, TX) by upward capillary action overnight using 20× SSC, cross-linked using a UV Stratalinker 2400 (Stratagene), and hybridized overnight at 50°C with DIG-labeled double-stranded DNA (dsDNA) probes specific for *glpK* and *glpA1*. PCRs for the generation of the probes were performed with the primers listed in Table S2 in the supplemental material and *Taq* DNA polymerase according to the supplier's recommendations with the following modifications: 3% (vol/vol) DMSO was included, and the 1× DIG deoxyribonucleoside triphosphate mixture (catalog no. 1277065; Roche) was supplemented with a solution of mixed deoxynucleotides (New England Biolabs) to 0.1 mM. For hybridization, membranes with the cross-linked RNA samples were equilibrated in high-sodium dodecyl sulfate (SDS) buffer (5× SSC, 2% [wt/vol] blocking reagent, 0.1% [wt/vol] *N*-lauroylsarcosine, 0.2% [wt/vol] SDS, 50% [wt/vol] formamide) (2 h, 50°C), followed by incubation with 100 ng labeled probe in 10 ml of high-SDS buffer (16 h, 50°C). Membranes were washed with 2× SSC supplemented with 0.1% (wt/vol) SDS (twice, for 5 min each time) and with 0.1× SSC supplemented with 0.1% (wt/vol) SDS (twice, for 15 min each time, at 50°C). Hybridization products were detected by a chemiluminescent (CSPD*) digoxigenin immunoassay (Roche).

Transcriptional reporter construction and assay. A plasmid-based reporter system was used to analyze transcription (10) from promoter regions upstream of *glpA1*, *glpK*, and *tnaA* that were fused to the *Haloferax alicantei*-derived *bgaH* gene encoding β-galactosidase (for details, see Tables S1 and S2 in the supplemental material). The promoter activity of each construct was determined by an assay of the β-galactosidase activity of log-phase cells as described previously (17). One unit of β-galactosidase activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol *o*-nitrophenyl-β-D-galactopyranoside (ONPG) $\cdot$ min^{-1} with a molar extinction coefficient for *o*-nitrophenol of 3,300 $M^{-1} \cdot cm^{-1}$.

HPLC analysis of glycerol and glucose. At various time points, culture broths (1 ml) of both the parent (H26) and *glpA1* mutant (KS11) strains growing on 5 mM Gly Glu MM (MM supplemented with glycerol and glucose at 5 mM each) were withdrawn and centrifuged (10 min at 10,000 × *g* and 4°C). Supernatant fractions were filtered and analyzed by high-performance liquid chromatography (HPLC) using a Bio-Rad HPX-87H column with a 4 mM H_2SO_4 eluent.

DNA sequencing. Sanger automated DNA sequencing was performed using an Applied Biosystems model 3130 genetic analyzer (ICBR Genomics Division, University of Florida).

RESULTS AND DISCUSSION

Bacterial G3PDH homologs in haloarchaea. Glycerol metabolism in *H. volcanii* requires the phosphorylation of glycerol to G3P by a bacterial protein-like glycerol kinase (encoded by *glpK*) (27). To analyze the subsequent step, the oxidation of G3P to DHAP, homologs of bacterial G3PDH enzymes known to catalyze this reaction were identified in *H. volcanii* and other haloarchaeal genomes. This included the identification of haloarchaeal homologs of all three subunits of the anaerobic G3PDH complex (GlpA, GlpB, and GlpC) of bacteria (see Fig. S1 in the supplemental material). Homologs of the G3PDH catalytic subunit A, GlpA, were also identified in archaea of

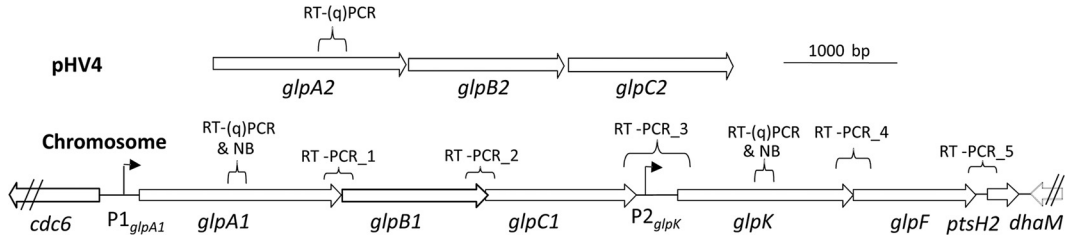

FIG. 1. Organization of glycerol metabolic genes on the *H. volcanii* DS2 genome. Shown are schematic representations of the *glpA2B2C2* genes on megaplasmid pHV4 and of the *glpA1B1C1*, *glpK*, *glpF*, and *ptsH2* genes on the main chromosome. Open reading frames *glpA1B1C1* (HVO_1538 to HVO_1540) and *glpA2B2C2* (HVO_A0269 to HVO_A0271) encode G3PDH homologs; *glpK* (HVO_1541) encodes glycerol kinase; *glpF* (HVO_1542) encodes a putative glycerol facilitator (GlpF) based on predicted membrane topology and common linkage to glycerol metabolic operons among diverse haloarchaea; and *ptsH2* (HVO_1543) encodes a homolog of phosphotransferase system (PTS) histidine-phosphorylatable Hpr proteins of bacteria (for reviews of bacterial GlpF and Hpr proteins, see references 29 and 11, respectively). Braces indicate annealing sites for primer pairs and regions amplified by RT-PCR. Annealing sites for primer pairs used in the construction of *glpA1*- and *glpK*-specific probes for Northern blotting (NB) and RT-qPCR are also indicated (for primer details, see reference 27). $P1_{glpA1}$ and $P2_{glpK}$, promoter regions upstream of *glpA1* and *glpK*, respectively. Open arrows represent coding regions for each open reading frame. Bar, 1,000 bp.

the classes *Thermoplasmata* and *Thermoprotei* (see Fig. S1 in the supplemental material). In contrast to the haloarchaea, the latter archaea do not encode homologs of the bacterial GlpB or GlpC proteins, which (in addition to GlpA) are essential for the anaerobic growth of *E. coli* on G3P (31). Thus, among the archaea, only the haloarchaea appear to encode bacterial protein-like G3PDH complexes in addition to homologs of bacterial glycerol kinase and PTS components, including a putative PEP:PTS-dependent DHA kinase (27).

Most haloarchaea encode two GlpA-related proteins (GlpA1 and GlpA2) and single GlpB and GlpC homologs. Typically, the haloarchaeal genes (*glpA1* and *glpA2*) encoding the GlpA homologs are located on the main chromosome, with *glpA1* organized in an apparent *glpA1 glpB1C2* operon and *glpA2* in a separate chromosomal region (see Fig. S1 in the supplemental material). *H. volcanii* is exceptional in that it harbors two apparent *glpABC* operons, with (i) *glpA1* located on the main chromosome upstream of *glpB1C1-glpK-glpF-ptsH2* and (ii) *glpA2* located on megaplasmid pHV4 upstream of *glpB2C2* (Fig. 1). While most of the haloarchaeal GlpA homologs cluster phylogenetically into distinct GlpA1 and GlpA2 lineages, the *H. volcanii* GlpA homologs cluster together in the haloarchaeal GlpA1 lineage (see Fig. S1). Consistent with this relationship, *H. volcanii* GlpA1 and GlpA2 are similar in amino acid length and harbor a C-terminal bacterioferritin-associated ferredoxin (BFD)-like [2Fe-2S] domain common to bacterial GlpA proteins (see Fig. S2 in the supplemental material). The GlpA2 homologs of other haloarchaea are missing the BFD-like domain. Although the physiological role of BFD domains remains unclear, this protein may serve as a general redox enzyme and/or a regulatory component of iron storage and mobilization (15). Thus, based on its genome sequence, *H. volcanii* harbors two separate *glpABC* operons that may encode functional G3PDH complexes.

Knockout of *glpA1* and *glpA2* from the *H. volcanii* genome. To investigate the function of haloarchaeal G3PDH homologs, *glpA1* and *glpA2* were targeted for markerless deletion from the genome of *H. volcanii* H26 (designated the wild-type strain throughout this study). Genes encoding GlpA homologs were selected for knockout, since subunit A is required for the catalytic activity of G3PDH in *E. coli* (9). Markerless deletion was confirmed by PCR, Southern blotting, and DNA sequence analysis (see Fig. S3 in the supplemental material).

Requirement of *glpA1* for glycerol metabolism. The *glpA1* (KS11) and *glpA2* (KS10) mutant strains were compared to the wild type with respect to growth on glycerol and glucose (Gly MM and Glu MM, respectively). Since G3PDH gene expression is often regulated by environmental conditions (14), strains were examined for growth on glycerol by using oxygen as well as dimethyl sulfoxide as a terminal electron acceptor. While all strains grew similarly to the wild type on glucose, and the *glpA2* mutant grew similarly to the wild type on glycerol, the *glpA1* mutant was unable to grow on glycerol (Fig. 2A). The *glpA1* mutant was complemented by expressing *glpA1* or *glpA2* in *trans* from a strong *Halobacterium salinarum* rRNA P2 promoter with the pHV2-based plasmid pJAM2696 or pJAM2711, respectively (Fig. 2A; see also Table S1 in the supplemental material). HPLC analysis of cell culture broth revealed that the *glpA1* mutant could not utilize glycerol and consumed only glucose during growth in a medium with glycerol and glucose (Fig. 2B). This contrasted with the behavior of wild-type cells, which utilized both glycerol and glucose, with an apparent preference for glycerol, as observed previously (27). Together, these results reveal that *glpA1* (like *glpK*-encoded glycerol kinase [27]) is needed for growth on glycerol and for glycerol metabolism. The ability of *glpA2* to *trans*-complement the *glpA1* mutant suggests that GlpA2 is a functional homolog of GlpA1. Since the genomic copy of *glpA2* is not required for growth on glycerol and does not compensate for the loss of *glpA1* when controlled by the *glp2* native promoter, it is likely that the genomic copy of *glpA2* is not transcribed under these conditions (even in the absence of *glpA1*). Thus, *glpA2* transcript levels were examined by RT-PCR for wild-type and mutant strains, including KS10 (Δ*glpA2*) and KS11 (Δ*glpA1*), with and without *trans*-complementation by *glpA2* (pJAM2711). Cells were grown on medium with glycerol and glucose; the latter carbon source was included to allow for the growth of KS11. With this approach, *glpA2* transcripts were readily detected in the *glpA1* mutant *trans*-complemented with *glpA2* (KS11/pJAM2711) but were not detected when *glpA2* was present only in a genomic copy or was deleted (H26, KS10, and KS11) (see Fig. S4 in the supplemental material). These

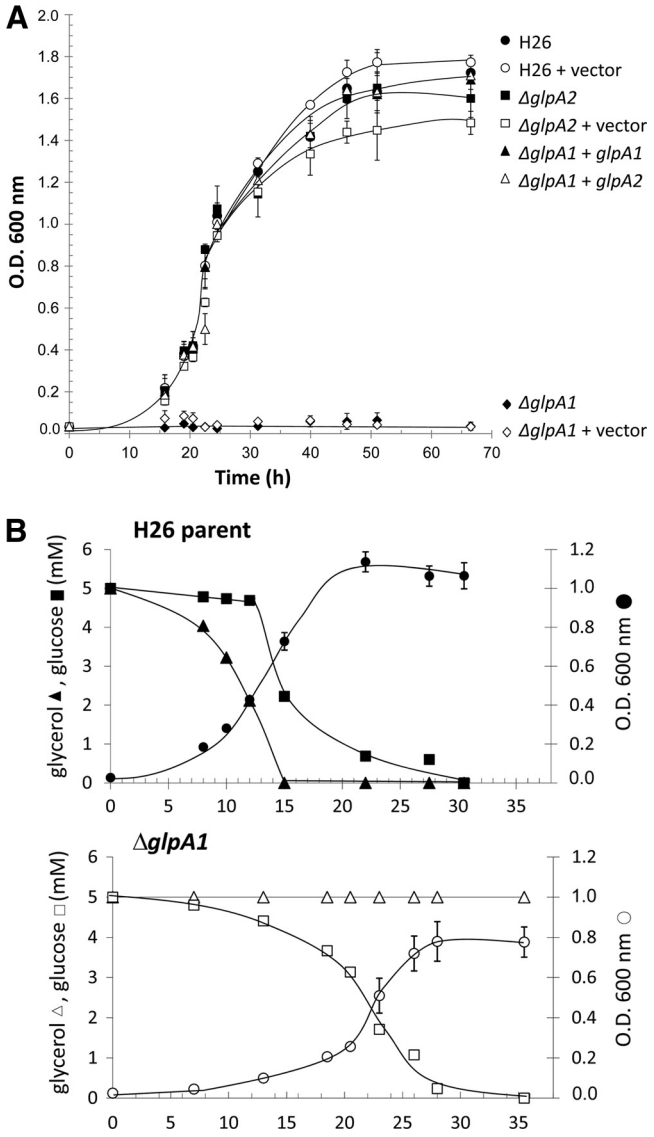

FIG. 2. Catabolism of glycerol requires the G3PDH homolog GlpA1 in *H. volcanii*. (A) Growth of *H. volcanii* strains on glycerol (Gly MM) with oxygen as a terminal electron acceptor. *H. volcanii* strains (indicated on the right) include the wild-type parent (H26) and the Δ*glpA1* (KS11) and Δ*glpA2* (KS10) strains with or without the vector control (pJAM202c) or a complementary plasmid (pJAM2696, providing *glpA1* in *trans*, or pJAM2711, providing *glpA2* in *trans*). Growth was monitored by the increase in the OD_{600}. Experiments were performed in triplicate, and the means ± standard deviations were calculated. A similar requirement of *glpA1* (and not *glpA2*) for growth on glycerol was observed when cells were grown anaerobically with DMSO as the terminal electron acceptor (data not shown). (B) Metabolism of glycerol by the *H. volcanii* wild-type strain (H26) compared to that by the *glpA1* mutant strain (KS11). *H. volcanii* strains were inoculated onto 5 mM Gly Glu MM and were monitored for growth (OD_{600}) as well as for the utilization of glucose and glycerol in the presence of oxygen as indicated. All experiments were performed at least in biological triplicate.

findings explain why *glpA1* (and not *glpA2*) is required for standard growth on glycerol and why *glpA2* complements the *glpA1* knockout when expressed in *trans* from a strong promoter. Whether *glpA2* is induced by other environmental conditions is unclear.

G3PDH activity levels are altered by the carbon source. To further investigate G3PDH, its specific activity was determined in lysates of *H. volcanii* cells (H26, the wild-type strain) grown to log phase on a medium containing glycerol, glucose, or glycerol plus glucose. G3PDH activity was 2.7-fold higher in cells grown on glycerol than in cells grown on glucose alone (Table 1). However, as with the *H. volcanii* glycerol kinase (27), the levels of G3PDH activity were not significantly reduced in cells grown with both glucose and glycerol. This contrasts with the findings for many bacteria and yeast that glycerol catabolism (including the levels of glycerol kinase and G3PDH activities) is substantially reduced by the addition of glucose to the growth medium (22, 28).

GlpA1 and GlpA2 are required for full G3PDH activity. We next examined whether *glpA1* and/or *glpA2* was responsible for the G3PDH activity observed. Cell lysates were prepared from *glpA1* and *glpA2* mutants (grown on glycerol and/or glucose) and were assayed for G3PDH activity compared to that for the wild type. Neither single knockout of *glpA1* nor single knockout of *glpA2* had a notable impact on the low levels of G3PDH activity detected in glucose-grown cells (Table 1). However, when cells were grown in the presence of glycerol, the G3PDH activity of the *glpA1* mutant remained low, at levels 3.7-fold lower than those for the wild-type strain under these growth conditions (Table 1). In contrast, knockout of *glpA2* had only a slight impact (a 1.4-fold reduction from the wild-type level) on the high-levels of G3PDH activity observed in cells grown on glycerol (with or without glucose) (Table 1). Both the *glpA1* and *glpA2* mutant strains were complemented by providing a copy of the respective gene in *trans*, confirming that the reductions in G3PDH activity observed on glycerol were not due to polar effects of the mutations (Table 1). The G3PDH activity of the *glpA1* mutant was also restored to wild-type levels by providing *glpA2* in *trans* (Table 1), again suggesting that *glpA2* is a functional analog of *glpA1*. To further investigate the roles of *glpA1* and *glpA2*, a double knockout of both *glpA1* and *glpA2*

TABLE 1. G3PDH-specific activity of the parent strain and mutant strains deficient in the synthesis of glycerol kinase or G3PDH subunit A homologs

Strain[a]	G3PDH sp act (mU · mg^{-1})[b] with the following medium:		
	Gly MM	Glu MM	Gly Glu MM
H26 (parent)	76 ± 10	28 ± 4	67 ± 10
Δ*glpA1* mutant	No growth	19 ± 1	18 ± 1
Δ*glpA2* mutant	55 ± 6	24 ± 2	47 ± 6
Δ*glpA1* Δ*glpA2* mutant	No growth	UD	UD
Δ*glpK* mutant	No growth	19 ± 4	21 ± 1
Δ*glpA1*/*glpA1* strain	70 ± 9	26 ± 6	68 ± 9
Δ*glpA1*/*glpA2* strain	67 ± 3	28 ± 5	67 ± 4
Δ*glpA2*/*glpA2* strain	73 ± 9	28 ± 5	72 ± 7
Δ*glpK*/*glpK* strain	72 ± 8	27 ± 3	67 ± 5

[a] The Δ*glpK* strain is deficient in the synthesis of glycerol kinase; the Δ*glpA1* and Δ*glpA2* strains are deficient in the synthesis of G3PDH subunit A homologs. Slashes are used to indicate strains with plasmids expressing *glpK*, *glpA1*, or *glpA2* in *trans*.

[b] G3PDH activity was determined for cell lysates as described in Materials and Methods. Cells were grown to log phase in minimal medium (MM) with Gly, Glu, or both as indicated. No growth, mutant strains that did not grow on Gly MM; UD, undetectable levels of activity. Experiments were performed in biological triplicate, and the means ± standard deviations were calculated. No activity was detected for controls with no substrate or with boiled cell lysates.

TABLE 2. Transcription from the *glpA1*, *glpK*, and *tnaA* promoter regions based on a β-galactosidase reporter gene

Medium and strain	Sp act of β-galactosidase (mU · mg^{-1})[a] with the following promoter[b]:				
	$P1_{glpA1}$ (310 bp)	$P2_{glpK}$ (354 bp)	P_{tnaA} (321 bp)	$P2_{rrnA}$ (551 bp)	Vector (none)
Gly MM					
H26 (parent)	310 ± 5	16 ± 2	ND	260 ± 10	8.1 ± 0.1
Δ*glpK* mutant	No growth	No growth	No growth	No growth	No growth
Δ*glpA1* mutant	No growth	No growth	No growth	No growth	No growth
Δ*glpR* mutant	300 ± 7	18 ± 2	ND	260 ± 3	8.7 ± 0.9
Glu MM					
H26 (parent)	38 ± 0.1	22 ± 0.7	ND	250 ± 7	7.3 ± 0.9
Δ*glpK* mutant	30 ± 1	25 ± 2	ND	250 ± 8	6.5 ± 0.7
Δ*glpA1* mutant	35 ± 3	21 ± 1	ND	260 ± 3	9.2 ± 1
Δ*glpR* mutant	36 ± 0.5	23 ± 0.6	ND	250 ± 4	8.3 ± 0.9
Gly Glu MM					
H26 (parent)	280 ± 8	14 ± 2	ND	260 ± 2	8.1 ± 0.05
Δ*glpK* mutant	28 ± 2	22 ± 3	ND	250 ± 5	8.3 ± 0.6
Δ*glpA1* mutant	270 ± 9	18 ± 0.5	ND	240 ± 6	7.3 ± 0.5
Δ*glpR* mutant	270 ± 8	19 ± 1	ND	250 ± 7	8.0 ± 0.5
Suc MM (H26 [parent])	ND	ND	38 ± 6	260 ± 20	8.0 ± 0.08
Suc Trp MM (H26 [parent])	ND	ND	1,700 ± 50	230 ± 30	7.4 ± 0.07

[a] Determined from the lysates of cells grown to log phase in minimal medium (MM) as indicated. No growth, mutant strains that did not grow on Gly MM; ND, not determined. Experiments were performed in biological triplicate, and the means ± standard deviations were calculated.

[b] The parental strain H26 and the Δ*glpK*, Δ*glpA1*, and Δ*glpR* mutant strains were transformed with a plasmid carrying the promoter region of *glpA1* ($P1_{glpA1}$) or *glpK* ($P2_{glpK}$) transcriptionally fused to the β-galactosidase *bgaH* reporter gene. The tryptophan-inducible promoter P_{tnaA} and the strong promoter $P2_{rrnA}$ were included for comparison. Promoter fusions included the start codon and genomic region immediately upstream of each target gene. The length of the promoter fusion is given in parentheses after the promoter designation. Plasmid vector pJAM2715, containing only a Shine-Dalgarno sequence upstream of *bgaH*, served as a negative control.

was constructed (KS12) and was found to be devoid of any detectable G3PDH activity, even when cells were grown in the presence of glycerol (Gly Glu MM) (Table 1). Thus, GlpA1 and GlpA2 are required for the full G3PDH activity of *H. volcanii*, and the levels of the GlpA1-dependent G3PDH activity are substantially increased by the addition of glycerol to the growth medium.

G3P is needed for enhanced levels of GlpA1-dependent G3PDH activity. Glycerol catabolism is often intricately coordinated by a number of mechanisms in prokaryotic and eukaryotic cells. In *E. coli*, the *glp* regulon (mediating glycerol and G3P catabolism) is controlled at the transcriptional level by anaerobic conditions, catabolite repression, and the inducer G3P, which binds the DeoR-type regulator GlpR and alleviates transcriptional repression of the *glp* regulon (14, 33). To improve our understanding of the inducers of glycerol catabolism in *H. volcanii* that may be responsible for the enhanced levels of GlpA1-dependent G3PDH activity observed during growth on glycerol (with or without glucose) compared to growth on glucose alone, the G3PDH activity of a *glpK* (glycerol kinase) mutant was determined. Consistent with the possibility that G3P regulates the levels of G3PDH produced in the cell, the *glpK* mutant (unable to synthesize G3P) displayed a lower level of G3PDH activity than the wild-type strain during growth in the presence of glycerol (Table 1). G3PDH activity was restored to wild-type levels in the *glpK* mutant by *trans*-complementation with *glpK* (Table 1). Thus, we speculate that G3P, generated by GlpK during growth in the presence of glycerol, alleviates the transcriptional repression of *glpA1* and enhances the levels of G3PDH in *H. volcanii*.

Intergenic regions upstream of *glpA1* and *glpK* can drive transcription. To further examine the regulation of glycerol catabolism in *H. volcanii*, genomic regions immediately upstream of the translational start codons of *glpA1* ($P1_{glpA1}$) and *glpK* ($P2_{glpK}$) (310 and 354 bp, respectively) were fused to the *H. alicantei bgaH* gene, encoding β-galactosidase. Transcription, driven by promoter elements within these regions, was monitored by an assay of β-galactosidase activity in lysates prepared from cells carrying these transcription fusions. With this approach, transcription from $P1_{glpA1}$ and $P2_{glpK}$ was found to be higher than that from the vector control, which retained a Shine-Dalgarno sequence but lacked promoter elements upstream of the *bgaH* reporter gene (Table 2). Most notable was transcription from $P1_{glpA1}$, which was 7-fold higher during growth on glycerol (with or without glucose) than during growth on glucose alone. In contrast, transcription from $P2_{glpK}$ was constitutive and at relatively low levels, only ~2-fold higher than that from the vector control (for all strains and conditions examined) (Table 2). The glycerol-responsive promoter $P1_{glpA1}$ was not as highly induced as the tryptophan-responsive *tnaA* promoter (P_{tnaA}) developed by Large et al. (20) for conditional expression of genes in *H. volcanii* (7-fold induction for $P1_{glpA1}$ versus 45-fold induction for P_{tnaA}) (Table 2). However, $P1_{glpA1}$ may serve as a nice complement to P_{tnaA} for experiments requiring differential gene regulation. When glycerol was included in the medium, expression from $P1_{glpA1}$ was comparable to that from *H. salinarum* $P2_{rrnA}$, used routinely for protein production in recombinant strains of *H. volcanii* (18, 30) (Table 2).

G3P is an inducer of *glpA1* transcription. To examine whether G3P is an inducer of G3PDH gene expression, transcription from $P1_{glpA1}$ was monitored in *glpK* and *glpA1* mutant strains. Wild-type cells and reporter gene constructs carrying $P2_{glpK}$ and $P2_{rrnA}$ promoter fusions were included along with the vector control for comparison. The rationale for using the *glpK* and *glpA1* mutants is that these strains are likely to differ in their cellular G3P levels during growth in the presence of glycerol. The *glpK* mutant is unable to convert glycerol to G3P (27), while the ability of the *glpA1* mutant to oxidize G3P to DHAP is impaired (Table 1). Consistent with the model that

G3P is an inducer of transcription from a promoter upstream of *glpA1*, our reporter assays revealed that transcription from $P1_{glpA1}$ in a *glpK* knockout strain was constitutive and was 10-fold lower than that of the wild type (and the *glpA1* mutant) when cells were grown in the presence of glycerol (Table 2). Expression from $P2_{glpK}$ and the $P2_{rrnA}$ control was also constitutive but was not altered by any of the genomic mutations or growth conditions examined (Table 2). Thus, in the absence of G3P (in the *glpK*-deficient strain), transcription from $P1_{glpA1}$ is reduced to constitutive and basal levels. These results are consistent with the *E. coli* model, in which G3P serves as the inducer for the glycerol metabolic operon (9). In *E. coli*, G3P relieves the transcriptional repression mediated by GlpR (21), thus allowing expression of the *glp* regulon, including the *glpD*, *glpTQ*, and *glpFKX* operons, when cells are grown in the absence of glucose and the presence of glycerol.

GlpR is not required for G3P induction of *glpA1* transcription. To directly examine whether *H. volcanii* GlpR modulates transcription from either $P1_{glpA1}$ or $P2_{glpK}$, the transcriptional reporter constructs of these promoter regions were analyzed in a *glpR* knockout strain (KS8) (Table 2). In contrast to the *E. coli* model, we do not predict repression of the *H. volcanii* glycerol metabolic operon by a DeoR/GlpR-type regulator. This hypothesis is based on our previous data (27), which demonstrate that during growth on glycerol, the single DeoR/GlpR-type regulator of *H. volcanii* controls both fructose and glucose metabolic enzymes through transcriptional repression of *pfkB* (encoding phosphofructokinase) and *kdgK1* (encoding 2-keto-3-deoxy-D-gluconate kinase) (25). In agreement with our prediction that *H. volcanii* GlpR is not required for the regulation of G3PDH gene expression, transcription from both $P1_{glpA1}$ and $P2_{glpK}$ was independent of the *glpR* mutation (Table 2). This apparent difference in the regulation of the glycerol metabolic operons between *H. volcanii* and *E. coli* is consistent with the distant phylogenetic relationship between these two microbes and their disparate habitats. Glycerol is a major source of carbon in many of the hypersaline environments where *H. volcanii* thrives, whereas it plays a more limited role in the ecosystems of *E. coli*. Furthermore, *H. volcanii* has an apparent preference for glycerol over glucose, while *E. coli* does not.

Transcriptional organization of the *glp* operon. Due to the close proximity of *glpA1B1C1*, *glpK*, *glpF*, and *ptsH2* on the chromosomes of *H. volcanii* (Fig. 1) and other haloarchaea, we investigated whether these genes formed an operon(s). Initial analysis was performed by RT-PCR using total RNA extracted from wild-type cells grown on glycerol (Gly MM). Primers were designed to anneal within the coding regions of neighboring genes, including *glpA1* and *glpB1* (13-bp overlap in the coding sequence), *glpB1* and *glpC1* (3-bp overlap in the coding sequence), *glpK* and *glpF* (4-bp intergenic region), *glpF* and *ptsH2* (2-bp overlap in the coding sequence), and *glpC1* and *glpK* (363-bp intergenic region) (Fig. 1); the latter primer pair is from our previous work (27). In each case, a single RT-PCR product with a molecular size and DNA sequence consistent with cotranscription of these neighboring genes was detected (Fig. 3A).

To further examine the transcripts generated from the glycerol metabolic operon, total RNA (extracted from wild-type cells grown on glucose with or without glycerol) was analyzed by Northern blotting using probes specific for *glpA1* and *glpK*. With this approach, *glpA1*-specific transcripts of ~4.3 kb, a size consistent with the *glpA1B1C1* coding region, were readily detected in cells grown on glycerol plus glucose (Fig. 3B). Less-prominent *glpA1*-specific transcripts of ~7.7 kb were also observed in these cells, suggesting that the *glpA1B1C1* operon is cotranscribed with its downstream gene neighbors (Fig. 3B). In addition to these *glpA1*-specific transcripts, *glpK*-specific transcripts of multiple lengths were identified in cells grown on glycerol and glucose, including highly abundant transcripts of ~2.5 kb as well as less abundant transcripts of ~4.3, 6.3, and 7.7 kb (Fig. 3B). Although most of the transcripts that hybridized to the *glpA1*- and *glpK*-specific probes were not observed

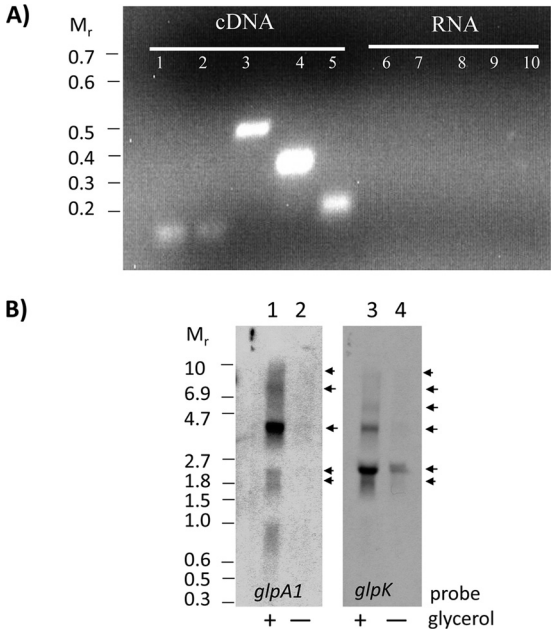

FIG. 3. The *H. volcanii* G3PDH and glycerol kinase genes required for glycerol catabolism are cotranscribed in an operon. (A) RT-PCR analysis of glycerol metabolic genes. *H. volcanii* RNA (total RNA isolated from the parent strain H26 grown aerobically on Gly MM) was analyzed by RT-PCR using primer pairs annealing to the 3′ and 5′ ends of *glpA1* and *glpB1* (lane 1), *glpB1* and *glpC1* (lane 2), *glpC1* and *glpK* (lane 3), *glpK* and *glpF* (lane 4), and *glpF* and *ptsH2* (lane 5) (primer pairs in Fig. 1 are numbered according to lane numbers here). Negative controls (lanes 6 to 10) included RNA that had not undergone reverse transcription for each PCR to confirm that the RT-PCR products (lanes 1 to 5, respectively) were not contaminated with genomic DNA. All RT-PCR products were sequenced to confirm specificity. The M_rs (in thousands) of DNA standards (Quick-Load 100-bp DNA ladder) are indicated on the left. (B) Northern blot analysis of the glycerol catabolic operon. Total RNA was isolated from *H. volcanii* H26 (parent) grown at 42°C (200 rpm) in the presence (Glu Gly MM [lanes 1 and 3]) or absence (Glu MM [lanes 2 and 4]) of glycerol and was analyzed by Northern blotting with DIG-labeled probes specific for *glpA1* (lanes 1 and 2) and *glpK* (lanes 3 and 4) as indicated. The M_rs (in thousands) of DIG-labeled RNA molecular weight marker I are indicated on the left. Detectable transcripts are indicated by arrows on the right. See Fig. 1 for details on primer and probe annealing sites as well as the organization of ORFs and intergenic spacers. The molecular sizes of genomic regions (ORFs and intergenic spacers) based on the DNA sequence of *H. volcanii* DS2 include the following: 7.7 kb for *glpA1B1C1-glpKF-ptsH2*, 6.2 kb for *glpA1B1C1-glpK*, 4.3 kb for *glpA1B1C1*, 3.2 kb for *glpC1-glpK*, 3.0 kb for *glpKF-ptsH2*, 2.6 kb for *glpKF*, and 1.5 kb for *glpK*.

in cells grown on glucose alone, the *glpK*-specific transcripts of ~2.5 kb were detected at low levels in glucose-grown cells. Based on its size, the latter transcript (present in cells grown in the presence or absence of glycerol) may be the cotranscript of *glpK* and *glpF* detected by RT-PCR (Fig. 3A), since these genes span 2.6 kb of the genome. Production of *glpKF* transcripts in the absence of glycerol may be physiologically advantageous, since both gene products (a glycerol kinase and a putative glycerol facilitator) are predicted to be required early in glycerol catabolism and produce the internal G3P needed to induce the system. The molecular mechanism(s) responsible for generating the *glpA1*- and *glpK*-specific transcripts of various lengths remains unclear. However, our results suggest that transcription of the glycerol metabolic operon of *H. volcanii* is driven by more than one promoter element (the G3P-inducible promoter $P1_{glpA1}$ and the constitutive promoter $P2_{glpK}$) and that the primary transcript(s) from this operon may be cleaved into shorter transcripts of differential stability. While mechanisms of RNA degradation in archaea have only recently been studied (13), examples of RNase-mediated endonucleolytic cleavage of primary transcripts into shorter transcripts with different half-lives are well characterized in bacteria (24).

Conclusions. Here we demonstrate that *H. volcanii* requires G3PDH encoded by an operon on the main chromosome for the catabolism of glycerol. Although two genomic regions (*glpA1B1C1* on the main chromosome and *glpA2B2C2* on megaplasmid pHV4) are predicted to encode homologs of all three subunits of the anaerobic G3PDH of bacteria, we demonstrate that G3P is dissimilated primarily through the GlpA1-containing G3PDH. GlpA2, though a functional complement to GlpA1 and required for the basal levels of G3PDH activity observed in a *glpA1* knockout strain, is not required for growth on glycerol. Interestingly, GlpA1 is needed for the enhanced levels of G3PDH activity observed when cells are grown on glycerol (with or without glucose) compared to growth on glucose alone. Promoter fusions to *bgaH* (Table 2) reveal that the genomic region upstream of *glpA1* ($P1_{glpA1}$) harbors a strong promoter element that is tightly controlled and is induced by growth in the presence of glycerol. This increase in $P1_{glpA1}$-mediated transcription is likely responsible for the differences observed between G3PDH activity during growth on glycerol and that in its absence. G3P appears to serve as the inducer of $P1_{glpA1}$-mediated transcription, based on the requirement of glycerol kinase (and not *glpA1*-encoded G3PDH) for this induction during growth on glycerol. In contrast to the *E. coli* model, this induction is not subject to regulation by a DeoR/GlpR-type repressor; instead, it is mediated by an uncharacterized protein in *H. volcanii*. Interestingly, transcription from the intergenic region between *glpC1* and *glpK* ($P2_{glpK}$) occurs only at basal levels and is not responsive to growth on glycerol. Further examination of the *glpA1B1C1*, *glpK*, *glpF*, and *ptsH2* genes by RT-PCR analysis and Northern blotting revealed that all six genes are cotranscribed with their neighboring genes. Distinct transcripts of various lengths are generated from this operon, not all of which are predicted based on transcription from $P1_{glpA1}$ and $P2_{glpK}$. Interestingly, we observed previously by RT-quantitative PCR (qPCR) that *glpA1*- and *glpK*-specific transcripts are at differential levels (78- and 9-fold, respectively) in glycerol-grown versus glucose-grown cells (27). The regulatory process(es) responsible for controlling the levels of these transcripts has yet to be fully elucidated. However, our current results reveal multiple mechanisms that may be utilized by the cell to control transcripts of this glycerol metabolic operon, including (i) G3P-inducible $P1_{glpA1}$-driven and constitutive $P2_{glpK}$-driven transcription and (ii) posttranscriptional processing of a primary transcript (spanning the entire 7.7-kb operon) into smaller transcripts that may be of differential stability.

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ERRATUM

Activity and Transcriptional Regulation of Bacterial Protein-Like Glycerol-3-Phosphate Dehydrogenase of the Haloarchaea in *Haloferax volcanii*

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Volume 193, no. 17, p. 4469–4476, 2011. Page 4469, column 2, lines 10 and 11: "GspA" should read "GpsA." Page 4471, column 1, line 14: "*glpB1C2*" should read "*glpB1C1*."