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GlpR Represses Fructose and Glucose Metabolic Enzymes at the Level of Transcription in the Haloarchaeon *Haloferax volcanii*▽†

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In this study, a DeoR/GlpR-type transcription factor was investigated for its potential role as a global regulator of sugar metabolism in haloarchaea, using *Haloferax volcanii* as a model organism. Common to a number of haloarchaea and Gram-positive bacterial species, the encoding *glpR* gene was chromosomally linked with genes of sugar metabolism. In *H. volcanii*, *glpR* was cotranscribed with the downstream phosphofructokinase (PFK; *pfkB*) gene, and the transcript levels of this *glpR-pfkB* operon were 10- to 20-fold higher when cells were grown on fructose or glucose than when they were grown on glycerol alone. GlpR was required for repression on glycerol based on significant increases in the levels of PFK (*pfkB*) transcript and enzyme activity detected upon deletion of *glpR* from the genome. Deletion of *glpR* also resulted in significant increases in both the activity and the transcript (*kdgK1*) levels of 2-keto-3-deoxy-D-gluconate kinase (KDGK), a key enzyme of haloarchaeal glucose metabolism, when cells were grown on glycerol, compared to the levels obtained for media with glucose. Promoter fusions to a β-galactosidase *bgaH* reporter revealed that transcription of *glpR-pfkB* and *kdgK1* was modulated by carbon source and GlpR, consistent with quantitative reverse transcription-PCR (qRT-PCR) and enzyme activity assays. The results presented here provide genetic and biochemical evidence that GlpR controls both fructose and glucose metabolic enzymes through transcriptional repression of the *glpR-pfkB* operon and *kdgK1* during growth on glycerol.

The archaeal basal transcriptional machinery closely resembles the eucaryal RNA polymerase II (RNAP II) apparatus. Along with a multisubunit RNAP (46), archaea encode two basal transcription factors, TATA-binding protein (TBP) and transcription factor B (TFB), which are homologs of the eucaryal TBP and general transcription factor TFIIB, respectively (5, 39). Although archaeal transcriptional components are fundamentally eukaryote-like in nature (35), the majority of candidate transcriptional regulators are homologous to bacterial activators and repressors (2, 26). Only a few archaeal candidate regulators resemble eukaryotic gene-specific transcription factors, one of the best characterized of which is GvpE, an activator of gas vesicle biosynthesis in haloarchaea which resembles the eukaryotic basic leucine zipper proteins (25, 31). While bioinformatics predicts many candidate archaeal regulators, only a limited number have been characterized at the molecular level, most of which are from hyperthermophiles (4, 13, 23, 24, 27, 42). Molecular data pertaining to haloarchaeal transcriptional regulation, specifically regulators of carbon utilization, are severely limited. Only a few global regulators, namely, transcription factors (8, 11, 36), have been implicated in regulating carbon utilization in haloarchaea. Specifically, in *Halobacterium salinarium*, pairs of general transcription factors TBP and TFB control gene clusters (8, 11), and transcription factor TrmB regulates diverse metabolic pathways in response to nutrient limitation (36). To our knowledge, no transcriptional regulators of sugar metabolism in the model haloarchaeon *Haloferax volcanii* have been characterized to date, although homologs of transcription factors can be predicted based on primary sequence. Furthermore, DeoR/GlpR-type regulators in archaea have yet to be characterized.

In this study, biochemical and genetic approaches were used to characterize GlpR, a putative transcriptional regulator of glycerol (Gly) and/or sugar metabolism in *H. volcanii*. Here, we present evidence that GlpR not only is autoregulatory but also regulates transcription of the downstream phosphofructokinase (PFK) gene *pfkB* as well as the distant chromosomal 2-keto-3-deoxy-D-gluconate kinase (KDGK) gene *kdgK1*. Taken together, our results provide the first example of a DeoR/GlpR-type repressor protein that controls key enzymes of sugar metabolism in haloarchaea and allow valuable insight into haloarchaeal metabolic transcriptional regulation.

MATERIALS AND METHODS

Materials. Biochemicals and enzymes used for PFK and KDGK activity analysis were purchased from Sigma-Aldrich (St. Louis, MO). the other organic and inorganic analytical-grade chemicals were from Fisher Scientific (Atlanta, GA) and Bio-Rad (Hercules, CA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IA). 2′-Deoxyuridine-5′-triphosphate coupled by an 11-atom spacer to digoxigenin (DIG) (DIG-11-dUTP), alkaline phosphatase-conjugated antibody raised against DIG, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD), and the other DIG-related biochemicals were from Roche Molecular Biochemicals (Indianapolis, IN). Positively charged membranes for Southern hybridization were from Ambion (Austin, TX). Phusion and *Taq* DNA polymerases, restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs (Ipswich, MA). Molecular biology-grade agarose used for the separation of DNA for Southern blotting and routine analysis was from Bio-Rad. Hi-Lo DNA standards were from Minnesota Molecular, Inc. (Minneapolis, MN).

Strains, media, and plasmids. Strains, oligonucleotide primers used for cloning, and plasmids are summarized in Table 1 and Table S1 in the supplemental

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TABLE 1. List of strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
Top10	F$^-$ *recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	Invitrogen
GM2163	F$^-$ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam13*::Tn*9 xylA5 mtl-1 thi-1 mcrB1 hsdR2*	New England Biolabs
H. volcanii		
DS70	Wild-type isolate DS2 cured of plasmid pHV2	32
H26	DS70 *pyrE2*	1
KS8	H26 *glpR* (devoid of GlpR)	This study
KS4	H26 *glpK* (devoid of GlpK)	38
KS10	H26 *glpK glpR* (devoid of GlpR and GlpK)	This study
Plasmids		
pTA102	Ap^r Nv^r; pGB70 containing *H. alicantei bgaH* derived from pMLH32	9
pTA131	Ap^r; pBluescript II containing P_{fdx}-*pyrE2*	1
pJAM202	Ap^r Nv^r; pBAP5010 containing $P2_{rrnA}$-*psmB-his*$_6$; β-His$_6$ expressed in *H. volcanii*	21
pJAM202c	Ap^r Nv^r; control plasmid derived from pJAM202	45
pJAM809	Ap^r Nv^r; pJAM202 containing $P2_{rrnA}$-*hvo1862-strepII* (KpnI site inserted upstream of StrepII coding sequence)	19
pJAM2676	Ap^r; pTA131 containing *glpR* with ~700 bp of genomic DNA flanking 5′ and 3′ of the *glpR* coding region	This study
pJAM2677	Ap^r; pJAM2676-derived *glpR* suicide plasmid	This study
pJAM2678	Ap^r Nv^r; pJAM202-derived plasmid containing $P2_{rrnA}$-*bgaH* from pTA102	This study
pJAM2682	Ap^r Nv^r; pJAM809 containing $P2_{rrnA}$-*glpR-strepII*	This study
pJAM2689	Ap^r Nv^r; pJAM2678 containing $P_{glpR\text{-}pfkB\text{-}188\ bp}$-*bgaH*	This study
pJAM2702	Ap^r Nv^r; pJAM2678 containing $P_{kdgK2\text{-}232\ bp}$-*bgaH*	This study
pJAM2703	Ap^r Nv^r; pJAM2678 containing $P_{HVO\_A0327\text{-}kdgK2\text{-}122\ bp}$-*bgaH*	This study
pJAM2705	Ap^r Nv^r; pJAM2678 containing $P_{kdgK1\text{-}89\ bp}$-*bgaH*	This study
pJAM2706	Ap^r Nv^r; pJAM2678 containing $P_{kdgK1\text{-}524\ bp}$-*bgaH*	This study
pJAM2714	Ap^r Nv^r; pJAM2678-derived plasmid devoid of $P2_{rrnA}$ from pTA102 and the Shine-Dalgarno site	This study
pJAM2715	Ap^r Nv^r; pJAM2678-derived plasmid devoid of $P2_{rrnA}$ from pTA102	This study

[a] Ap^r, ampicillin resistance; Nv^r, novobiocin resistance.

material. *Escherichia coli* Top10 was used for routine recombinant DNA experiments. *H. volcanii* strains were transformed (7) using plasmid DNA isolated from *E. coli* GM2163. Liquid cultures were aerated at 200 rpm. *E. coli* strains were grown at 37°C in Luria-Bertani medium supplemented with 100 mg per liter ampicillin as needed. *H. volcanii* strains were grown at 42°C in various media, including Casamino Acids (CA), yeast extract-peptone-Casamino Acids (YPC), YPC supplemented with glucose (Glu) or fructose (Fru), and minimal medium (MM) supplemented with glycerol (Gly), Glu, Fru, and various combinations of these carbon sources. Medium formulae were according to *The Halohandbook* (10), with the following exception: Glu, Fru, and/or Gly was included at 20 mM each where indicated. Media were supplemented as needed with 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). 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 enzyme activity and RNA extraction experiments, cells were freshly inoculated from −80°C glycerol stocks onto appropriate agar-based media on plates. Cells were twice subcultured and used as an inoculum for final analysis under various conditions as described below. Each subculture was inoculated to a final optical density at 600 nm (OD_{600}) of 0.03 to 0.04. For PFK and KDGK 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} (where 1 OD_{600} unit equals approximately 1×10^9 $CFU \cdot ml^{-1}$ for all strains used in this study). All experiments were performed at least in triplicate.

DNA isolation and analysis. DNA was separated by electrophoresis using 0.8% or 2% (wt/vol) agarose gels in 1× TAE electrophoresis buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5). Gels were stained with ethidium bromide at 0.5 $\mu g \cdot ml^{-1}$ and photographed with a mini-visionary imaging system (Fotodyne, Hartland, WI). The sizes of the DNA fragments were estimated using Hi-Lo DNA molecular weight markers. Plasmid DNA was isolated from *E. coli* strains by use of a QIAprep spin miniprep kit (Qiagen, Valencia, CA). PCR products were purified by MinElute (Qiagen) prior to modification by restriction enzymes (BamHI, HindIII, KpnI, NdeI, BlpI, or XbaI) or T4 DNA polynucleotide kinase. For rapid PCR screening, template DNA was extracted from *H. volcanii* mutant and parent strains and recombinant *E. coli* Top10 as described previously (45). For Southern blotting, *H. volcanii* genomic DNA was isolated from 5-ml cultures by DNA spooling (10), subjected to restriction enzyme digestion using MluI and HpaI, transferred to a nylon membrane, hybridized with a DIG-labeled probe specific for a 700-bp region flanking the 5′ end of the target coding region (primers listed in Table S1 in the supplemental material), and detected by CSPD-mediated chemiluminescence as described previously (45).

PCRs. High-fidelity double-stranded DNA used for the construction of plasmids listed in Table 1 was generated by PCR using Phusion DNA polymerase. *Taq* DNA polymerase was used for screening of mutant strains and for the generation of the DIG-labeled double-stranded DNA probes that were used for Southern blotting. All PCRs were performed according to the instructions of the suppliers, with the following modifications: 3% (vol/vol) DMSO was included as needed and 0.1 mM deoxyribonucleoside triphosphate mix was added to the standard DIG-labeling reaction mixture, which included 1× DIG deoxyribonucleoside triphosphate (catalog no. 1277065; Roche). Primer pairs and template DNA used for the PCRs are outlined in Table S1 in the supplemental material. PCR was performed using an iCycler or GeneCycler (Bio-Rad Laboratories), and products were analyzed by DNA electrophoresis.

Chromosomal knockout of *glpR*. The open reading frame (ORF) (HVO_1501; *glpR*) encoding a putative DeoR/GlpR repressor protein was targeted for markerless deletion from the chromosome of *H. volcanii* H26 using the *pyrE2*-based "pop-in/pop-out" method (1, 6). Colonies were screened for the absence of a 750-bp PCR product by use of internal primers specific for the coding region of the gene (the Negative-Forward and Negative-Reverse primers) and confirmed to be mutant strains by Southern blotting and PCR with the primer pair comprising Confirm-Forward and Confirm-Reverse, which anneal 100 bp upstream and downstream of the genomic region cloned into suicide plasmid pJAM2677.

The products of the latter PCR were sequenced to further confirm mutant strain fidelity (as described below). The primers used for the cloning and screening of mutant colonies are provided in Table S1 in the supplemental material.

PFK and KDGK activity assays. Exponential-growth-phase cells 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 × 20 s at 140 W). Debris was removed by centrifugation (10 min, 12,000 × *g*, 4°C). Protein concentration was estimated using the Bradford assay, with bovine serum albumin as a standard. PFK and KDGK activity assays were carried out as previously detailed (20), with the following exceptions: all enzyme activities were carried out aerobically at 37°C in a 96-well microplate reader filled with 0.1-ml assay mixtures. It was ensured that in coupled enzymatic assays, the auxiliary enzymes were not rate-limiting. Background change in absorbance for reaction mixtures containing no substrate was subtracted from the value for reactions in which substrate was included to yield the overall change in absorbance. Reaction mixtures containing boiled enzyme and no NADH were also included as controls. All experiments were performed in biological triplicate, and the means ± standard deviations (SD) of the results were calculated. One unit (U) of enzyme activity is defined as 1 μmol substrate consumed or product formed per min.

1-Phosphofructokinase (EC.2.7.1.56) specific activity was determined at 37°C by measuring the ATP-dependent formation of fructose-1,6-bisphosphate (FBP) from fructose-1-phosphate (F1P), which was coupled to the oxidation of NADH via FBP aldolase, triosephosphate isomerase (TIM), and glycerol-3-phosphate dehydrogenase (G3PDH). The assay mixture contained 100 mM Tris-HCl at pH 8.5 with 30 mM $MgCl_2$, 1 M KCl, 10 mM F1P sodium salt, 2 mM ATP, 0.3 mM NADH, 0.54 U FBP-aldolase, 2 U TIM, 0.34 U G3PDH, and cell extract (1 to 3 μg protein).

2-Keto-3-deoxy-D-gluconate kinase (EC 2.7.1.45) specific activity was determined at 37°C by measuring the ATP- and gluconate-dependent formation of pyruvate, which was coupled to the oxidation of NADH via lactate dehydrogenase (LDH). The assay mixture contained 100 mM Tris-HCl at pH 8.5 with 1 M KCl, 10 mM $MgCl_2$, 2 mM ATP, 0.3 mM NADH, 10 mM sodium gluconate, 11 U LDH, and cell extract (5 μg protein).

RNA purification and analysis. Total RNA was isolated from *H. volcanii* parent H26 and *glpR* mutant KS8 strains (exponential phase; OD_{600}, 0.3 to 0.5) 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 modifications: 3 U enzyme was added per 1 μg RNA, and the mixture was incubated for 45 min at room temperature. The integrity of the RNA was determined by agarose gel electrophoresis. RNA concentration was determined by A_{260} measurement using a Bio-Rad SmartSpec 3000 instrument.

Reverse transcription-PCRs (RT-PCRs) and quantitative RT-PCRs (qRT-PCRs) were performed using *H. volcanii* total RNA as a template (0.1 μg), appropriate primers (see Table S1 in the supplemental material), iQ SYBR green supermix (Bio-Rad), and an iCycler (Bio-Rad). RNA was reverse transcribed into cDNA using iSCRIPT (Bio-Rad) according to the manufacturer's instructions. After cDNA synthesis (25°C for 5 min, 42°C for 30 min, and 85°C for 5 min), qRT-PCR mixtures were preheated to 95°C (4 min), followed by 40 amplification cycles consisting of denaturation (95°C, 30 s), annealing (temperatures are listed in Table S1 in the supplemental material; 1 min), and elongation (72°C, 17 s). For RT-PCR, the reaction mixture was preheated to 95°C (4 min), followed by 35 amplification cycles consisting of denaturation (95°C, 30 s), annealing (55°C, 1 min), and elongation (72°C, 19 s), after which a final extension was performed at 72°C (10 min). For each primer pair, negative and positive controls were included to exclude genomic DNA contamination and confirm primer pair function, respectively. For the controls, the reactions were identical, with the following exceptions: the sample was maintained on ice during the reverse transcription step for the negative control, and *H. volcanii* genomic DNA prepared as previously described (29) was used as a template for the positive control. The products from the (q)RT-PCRs were sequenced as described below to confirm specificity.

Transcriptional reporter construction and assay. A plasmid-based reporter system in which the promoter regions of *glpR-pfkB* (HVO_1501-HVO_1500), *kdgK1* (HVO_0549), and *kdgK2* (HVO_A0328) were fused to the *Haloferax alicantei*-derived *bgaH* open reading frame encoding β-galactosidase was used to analyze transcription (9). The *bgaH* gene was amplified from pTA102 (primers are provided in Table S1 in the supplemental material) and cloned into pJAM202 using NdeI and BlpI, which fused the *bgaH* gene downstream of the strong rRNA P2 promoter of *Halobacterium cutirubrum* to generate pJAM2678. The regions upstream of *glpR-pfkB*, *kdgK1*, and *kdgK2* were amplified from *H. volcanii* genomic DS70 DNA using the primers listed in Table S1 in the supplemental material and fused with *bgaH* using XbaI and NdeI to generate pJAM2689 (188-bp *glpR-pfkB* promoter region), pJAM2705 (89-bp *kdgK1* promoter region), pJAM2706 (524-bp *kdgK1* promoter region), pJAM2702 (232-bp *kdgK2* promoter region), and pJAM2703 (122-bp HVO_A0327-*kdgK2* promoter region) (where base pair number represents the region upstream of the translational start codon of the first gene listed for each construct). Promoters were predicted upstream of *glpR-pfkB*, *kdgK1*, and *kdgK2* according to the methods described by Schneider et al. (37). Plasmid controls pJAM2714 and pJAM2715 were constructed using pJAM2678 by removal of the *H. cutirubrum* rRNA P2 promoter and the Shine-Dalgarno site (using XbaI and NdeI) and through removal of only the rRNA P2 promoter (using XbaI and BamHI), respectively.

The promoter activity of each construct was assessed quantitatively by assaying β-galactosidase activity as previously described (18). Briefly, cells were grown in 3 ml of appropriate media and harvested at exponential growth (0.3 to 0.5 OD_{600} units) by centrifugation (15 min, 6,000 × *g*, 4°C). Cell pellets were washed once in buffer B (50 mM Tris-HCl at pH 7.2 with 2.5 M NaCl, 10 μM $MnCl_2$), resuspended in 300 μl of *bgaH* buffer (buffer B with 0.1% [wt/vol] β-mercaptoethanol), and lysed using 150 μl of 2% Triton X-100. Debris was removed by centrifugation (10 min, 6,000 × *g*, 4°C), and the protein concentration in the cell extract was estimated using the Bradford assay (as above). Cells with promoter fusion constructs were assayed at 25°C for β-galactosidase specific activity by measuring the increase in absorbance at 405 nm due to the liberation of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The assay mixture (100 μl) contained 20 μl of cell lysate (3 to 5 μg protein), 70 μl of *bgaH* buffer, and 2.66 mM ONPG (stock solution with 8 mg · ml^{-1} in 100 mM potassium phosphate buffer, pH 7.2). Negative controls included cells carrying pJAM2714 and pJAM2715 (the *bgaH* reporter plasmids devoid of promoter elements). Background values in which no substrate (ONPG) was added to the reaction mixture were subtracted from the value for reaction mixtures containing substrate for each lysate tested. All experiments were performed in biological triplicate, and the means ± standard deviations (SD) of the results were calculated. One unit of β-galactosidase activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol ONPG · min^{-1} with a molar extinction coefficient for *o*-nitrophenol of 3,300 $M^{-1} \cdot cm^{-1}$.

DNA sequencing. The specificity of all PCR and (q)RT-PCR products, including DNA cloned into plasmids listed in Table 1 and amplified from the knockout region of mutant strains, was confirmed by Sanger automated DNA sequencing using an Applied Biosystems model 3130 genetic analyzer (ICBR Genomics Division, University of Florida).

RESULTS AND DISCUSSION

Identification of GlpR as a putative transcriptional repressor of metabolic enzymes. We previously demonstrated that *H. volcanii* exhibits glycerol-mediated catabolite repression of glucose metabolism when grown on minimal media containing both glycerol and glucose (38). In order to provide further insight into the central metabolism of haloarchaea, the *H. volcanii* genome (17) was searched for ORFs encoding proteins with DNA-binding domains that clustered near sugar metabolic operons. One such ORF (HVO_1501; designated *glpR*), when searched against the NCBI protein database, clustered with the DeoR/GlpR family of transcriptional regulators of sugar metabolism (COG1349) (see Fig. S1A in the supplemental material). The DeoR/GlpR protein family is widespread among bacteria, and its members often serve as transcriptional repressors (3, 16, 28, 34, 41, 44) or activators (14) of either sugar or nucleoside metabolism. DeoR/GlpR-type repressors are composed of approximately 250 amino acids, possess a helix-turn-helix DNA-binding motif near the amino terminus, and generally bind a sugar phosphate effector molecule of the relevant metabolic pathway in the C-terminal portion of the protein, which can also contain oligomerization domains (43). In *H. volcanii* as well as some haloarchaea and many Gram-positive bacterial species, *glpR* clusters on the genome with *pfkB*, encoding PFK (see Fig. S1B in the supplemental material), a key enzyme of fructose metabolism in haloarchaea

such as *H. volcanii* (12, 20). Dendrogram analysis revealed that *H. volcanii* GlpR clusters to the DeoR/GlpR family of transcriptional regulators, with closest relationship to uncharacterized proteins of haloarchaea and firmicutes (see Fig. S1B and S1C in the supplemental material). This result suggested that GlpR may be involved in regulating sugar metabolic enzymes at the level of transcription in *H. volcanii* and was thus targeted for further analyses (see below).

Transcripts encoding GlpR and PFK are under the control of a common promoter and are repressed by glycerol. In haloarchaea, PFK is involved in the metabolism of fructose through a modified Embden-Meyerhof-Parnas (EMP) pathway (12). Due to the close proximity of *glpR* and *pfkB* on the chromosome (4-bp overlap in coding sequence) (Fig. 1A), we investigated whether these genes were cotranscribed in an operon. RT-PCR was performed using primers designed to amplify a portion of each coding region (Fig. 1A). A single PCR product of expected size (0.2 kb) was detected using synthesized cDNA (Fig. 1B) and was further confirmed by DNA sequencing. No product was detected in the negative-control reaction mixture containing RNA as a template (Fig. 1B). Thus, *glpR* and *pfkB* are linked at the level of transcription.

Since *glpR* and *pfkB* are cotranscribed and PFK activity is largely fructose inducible and to some extent glucose inducible in haloarchaea (20), we investigated whether this regulation was controlled at the level of the *pfkB*-specific transcript and whether *glpR* was also induced by sugars. RNA was extracted from parent H26 grown in minimal media supplemented with different carbon sources, including fructose, glucose, and glycerol and various combinations thereof, and was subjected to qRT-PCR using primers specific for the 200-bp coding region of *glpR* and *pfkB* (see Table S1 in the supplemental material). Absolute quantification was performed for each transcript. In addition, a transcript specific for the ribosomal protein L10 gene (*ribL*) was used as an internal control based on previous studies (6a) and our confirmation by qRT-PCR that the *n*-fold value for induction of transcripts specific for *ribL* was close to 1.0 when parent H26 was grown in minimal media. With this approach, transcripts were found to be highly upregulated for both *glpR* (>20-fold ± 7-fold) and *pfkB* (>10-fold ± 3-fold) in the presence of fructose, regardless of glycerol supplementation, compared to the levels obtained with glycerol alone (Fig. 1C). Transcripts for *glpR* were also upregulated (>10-fold ± 4-fold) during growth on glucose, regardless of glycerol supplementation, compared to the levels obtained with glycerol alone (Fig. 1C). Thus, our data reveal that *glpR* and *pfkB* are cotranscribed from a common promoter and that the transcript levels of this operon are increased by fructose and, to a lesser extent, by glucose. These results are consistent with the observed sugar-dependent alterations in PFK activity for *Halococcus saccharolyticus* (20), in which the addition of fructose to peptide-rich media stimulated the level of PFK activity and suggest that PFK is regulated at the level of transcription in haloarchaea.

GlpR represses PFK transcription during growth on glycerol. To analyze the role of GlpR in regulating sugar metabolism, the *glpR* gene (HVO_1501) was targeted for knockout in *H. volcanii*. A markerless knockout strategy was used with the H26 Δ*pyrE2* parent strain as previously described (1, 6) to generate *glpR* mutant strain KS8. Gene deletion was confirmed by PCR, Southern blotting, and sequencing analysis (see Fig. S2 in the supplemental material). qRT-PCRs were used to compare the transcript levels of KS8 to those of parent strain H26 on various minimal media. With this approach, a *pfkB*-specific transcript was found to be significantly upregulated by the *glpR* knockout (10- to 12-fold) compared to the level for parent H26 when cells were grown in the presence of glycerol as the sole carbon source (Fig. 1D). This was complemented at least in part by providing a copy of *glpR* in *trans*, thus ruling out polar effects of the markerless deletion of *glpR* on the *glpR-pfkB* operon (Fig. 1D). In contrast to growth on glycerol alone, the *glpR* knockout had little if any impact on *pfkB*-specific-transcript levels when cells were grown in the presence of fructose with or without glycerol (Fig. 1D). Transcript levels remained high for *pfkB* in fructose-containing media for all strains analyzed (Fig. 1C) and were not significantly altered by the *glpR* mutation (Fig. 1D). Thus, while GlpR was required for the glycerol-mediated repression of *pfkB*-specific transcripts, GlpR was not needed for the high-levels of the *pfkB*-specific transcript present when cells were grown on fructose.

On the basis of the qRT-PCR findings showing that GlpR may serve as a repressor of *pfkB*-specific transcription in the absence of fructose and presence of glycerol, PFK activity in both parent and *glpR*-deficient strains was tested to determine if enzyme activity was altered. Consistent with the qRT-PCR results, PFK activity was increased in wild-type cells grown in media supplemented with fructose (versus media with glycerol alone) (Fig. 2). Also consistent with our qRT-PCR results, PFK specific activity was significantly increased by the *glpR* knockout (2-fold compared to the levels for the parent and *glpR*-complemented strains) when cells were grown on media with glycerol alone (Fig. 2). Deletion of *glpR* also resulted in a 1.5-fold increase in PFK activity compared to the levels for the parent and *glpR*-complemented strains when cells were grown in the presence of both fructose and glycerol (Fig. 2). In contrast to media with glycerol (with or without fructose), PFK activity was decreased during growth on fructose by deletion of *glpR* (Fig. 2). The reason for the latter finding remains to be determined but does not appear to be at the level of transcription, based on our qRT-PCR results.

The PFK activity of *H. volcanii* was also measured after growth on peptide-rich YPC medium with or without fructose or glucose. Similar to what was observed for minimal media, PFK activity was reduced when YPC was not supplemented with hexose sugars (see Fig. S3 in the supplemental material). GlpR, however, was not required for this decrease, which is in contrast to the GlpR-dependent reduction in PFK activity observed when sugars were excluded from glycerol minimal medium (Fig. 2; see also Fig. S3 in the supplemental material). It should be noted that the range of PFK activity values determined for the *H. volcanii* strains under the various conditions (140 to 650 mU · mg^{-1}) was in agreement with PFK activities reported for other haloarchaea (22 mU · mg^{-1} for *H. saccharolyticus* [20] and 1,300 mU · mg^{-1} for *Haloarcula vallismortis* [33] in peptide media with 25 to 28 mM fructose).

To determine whether the *glpR-pfkB* operon is regulated by GlpR at the level of transcription, (i) the 188-bp genomic region upstream of the start codon of *glpR* was fused to the coding region of the *H. alicantei bgaH* reporter, and (ii) the β-galactosidase activity of this reporter in parent and *glpR*

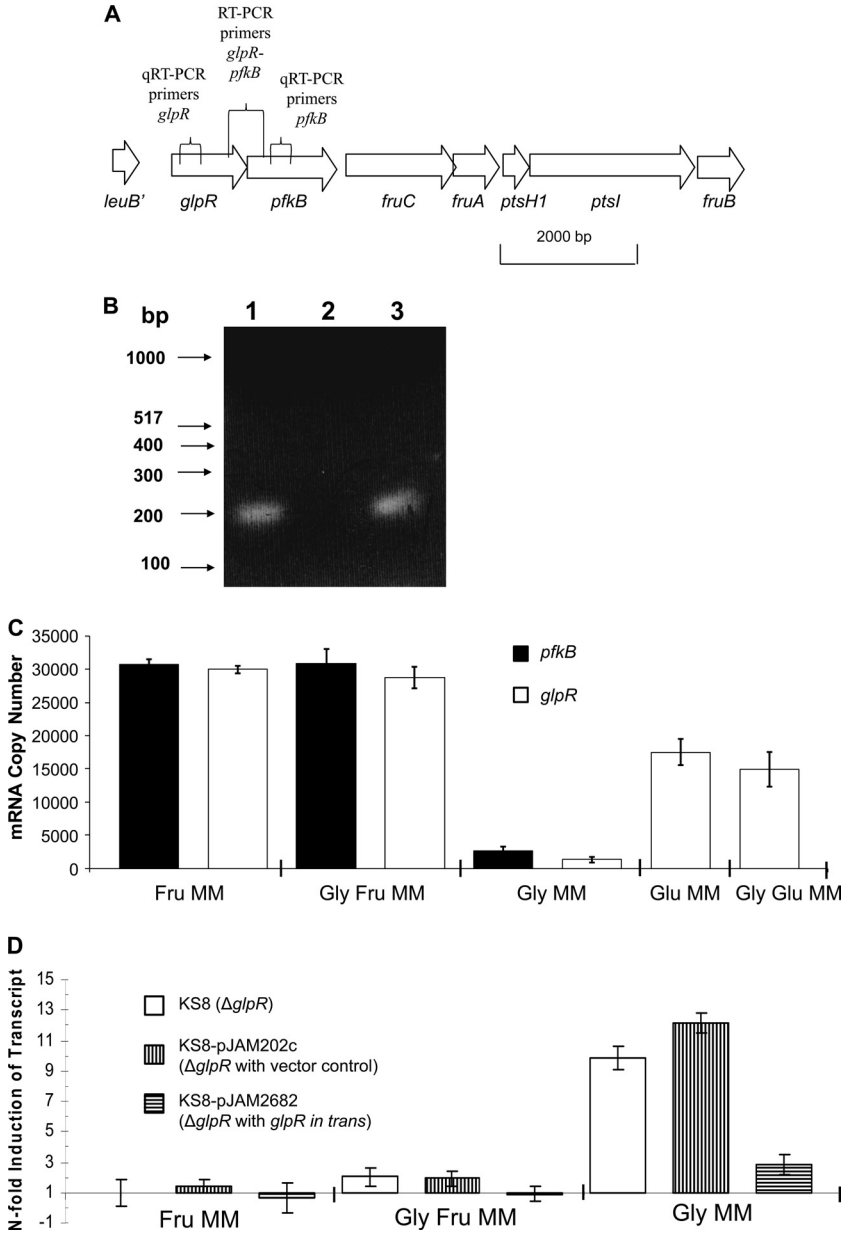

FIG. 1. Genomic organization and transcript analysis of *pfkB* encoding phosphofructokinase (PFK) and *glpR* encoding GlpR, a putative transcriptional repressor of the DeoR/GlpR family in *H. volcanii*. (A) Schematic representation of *glpR* and *pfkB* on the *H. volcanii* genome and locations of annealing sites (represented as vertical lines) for (q)RT-PCR primers. (B) qRT-PCR reveals that *pfkB* and *glpR* are cotranscribed from a common promoter. Total RNA from parent H26 was extracted and reverse transcribed to generate cDNA, which was used as a template for PCR (lane 1). RNA which had not undergone reverse transcription was used as a negative-control template for PCR (lane 2). Genomic DNA was used as a positive-control template for PCR (lane 3). Quick Load DNA markers (100 bp) and molecular sizes are indicated on the left. (C) Absolute quantifications of *pfkB*- and *glpR*-specific transcripts reveal increased transcript levels for both genes in the presence of fructose and glucose. Transcript levels were derived from qRT-PCR. (D) Relative quantifications of transcript levels specific for *pfkB* in *glpR* mutant KS8, KS8 with a plasmid vector control (pJAM202c), and KS8 with *glpR* in *trans* (pJAM2682) compared to the level for parent H26. PFK transcript is upregulated in the absence of fructose (in Gly) in KS8 compared to the level for H26. Transcript levels were derived by qRT-PCR. Calculations are based on the *n*-fold value for induction of transcript levels in KS8 (and subsequent control and complementary strains) compared to the level for parent H26. Results were normalized to the level for the internal control, *ribL*. Experiments were performed in triplicate, and the means ± SD were calculated.

mutant strains grown on various carbon sources was monitored. With this approach, significant and comparable levels of reporter activity were detected for both parent and *glpR* mutant strains when cells were grown in the presence of fructose, regardless of glycerol supplementation (Table 2). Under these conditions, the β-galactosidase specific activity measured for the *glpR-pfkB* promoter was 25 to 34 mU · mg^{-1}, compared to the levels for the negative controls, which lacked promoter elements (less than or equal to 12 mU · mg^{-1} for all conditions tested) (Table 2). In contrast, when cells were grown on glyc-

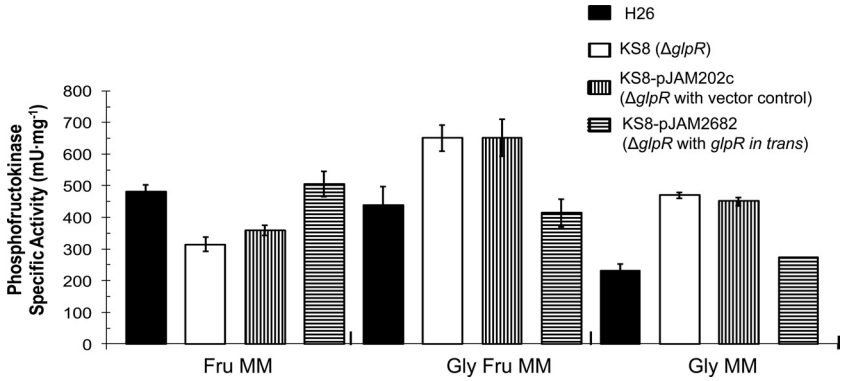

FIG. 2. PFK activity increases when cells are grown on glycerol minimal medium after deletion of *glpR*. Specific activity of PFK was determined from cell lysates of *H. volcanii* H26 (parent), KS8 (*glpR* mutant), KS8-pJAM202c (*glpR* mutant with vector control), and KS8-pJAM2682 (*glpR* mutant with *glpR* in *trans*) cells grown to log-phase in Fru, Gly-Fru, and Gly as indicated. Experiments were performed in biological triplicate, and the means ± SD were calculated.

erol alone, the promoter activity of the *glpR-pfkB* operon was reduced in parent strain H26 to levels comparable to that for the vector control, while deletion of *glpR* increased promoter activity to levels similar to those obtained with growth on fructose (Table 2). Overall, the data obtained with the promoter fusions are consistent with the qRT-PCR and PFK specific activity data and reveal that GlpR is required for transcriptional repression of the *glpR-pfkB* operon during growth on glycerol minus fructose, possibly by interacting with promoter elements within the 188-bp region upstream of this operon. The results also reveal that the *glpR-pfkB* promoter is relatively moderate at driving the expression of heterologous genes such as *bgaH* compared to the strong and constitutive *H. cutirubrum* rRNA P2 promoter, which reached levels of up to 260 mU · mg^{-1} of β-galactosidase activity for the reporter fusion (Table 2).

GlpR represses KDGK transcription during growth on glycerol. To address whether GlpR is involved in the repression of other sugar catabolic pathways, qRT-PCR primers were designed for analysis of the transcript levels of genes encoding homologs of KDGK, including chromosomally located *kdgK1* (HVO_0549) and pHV4-carried *kdgK2* (HVO_A0328) (Fig. 3A). In haloarchaea, KDGK is involved in the metabolism of glucose through a modified Entner-Doudoroff pathway (12). In *H. volcanii*, both *kdgK1* and *kdgK2* are predicted to encode active KDGK enzymes based on their conservation of active-site residues and their close relationship to KDGKs from other microbes that have been characterized at the biochemical level (see Fig. S4 in the supplemental material). Since KdgK1 and KdgK2 are close homologs (62% identity and 74% similarity in primary amino acid sequence), DNA sequencing was used to confirm qRT-PCR product specificity. On the basis of qRT-PCRs, the transcripts of both *kdgK* genes were significantly upregulated (4- to 12-fold) when parent strain H26 was grown on media containing glucose (with or without glycerol) compared to the levels obtained with glycerol alone (Fig. 3B).

TABLE 2. Transcription of a *bgaH* β-galactosidase reporter gene from *glpR-pfkB*, *kdgK1*, and *kdgK2* promoters in parent and *glpR* mutant strains grown on various carbon sources

Minimal medium	Mutation	β-Galactosidase sp act (mU/mg)[a] for indicated promoter						
		glpR-pfkB (188 bp)	*kdgK1* (524 bp)	*kdgK1* (89 bp)	HvoA0327-*kdgK2* (122 bp)	*kdgK2* (232 bp)	P2 *rmA* (551 bp)	None (Shine-Dalgarno site only)
Gly	None	9.2 ± 2	160 ± 3	180 ± 3	85 ± 0.01	14 ± 0.01	260 ± 10	8.1 ± 0.1
	Δ*glpR*	**32 ± 3**	**270 ± 10**	**250 ± 8**	99 ± 0.8	14 ± 0.01	250 ± 1	8.2 ± 0.2
Fru	None	27 ± 1	ND	ND	ND	ND	160 ± 7	8.2 ± 0.1
	Δ*glpR*	25 ± 0.3	ND	ND	ND	ND	150 ± 5	7.9 ± 0.2
Gly-Fru	None	27 ± 0.2	ND	ND	ND	ND	190 ± 10	7.3 ± 0.9
	Δ*glpR*	34 ± 0.1	ND	ND	ND	ND	180 ± 9	8.2 ± 0.2
Glu	None	ND	300 ± 4	280 ± 0.01	190 ± 0.01	41 ± 0.1	250 ± 7	7.3 ± 0.9
	Δ*glpR*	ND	250 ± 2	280 ± 3	160 ± 0.01	39 ± 0.02	250 ± 0.01	12.0 ± 0.02
Gly-Glu	None	ND	230 ± 5	200 ± 0.01	150 ± 4	33 ± 0.01	260 ± 7	8.1 ± 0.05
	Δ*glpR*	ND	300 ± 4	280 ± 1	160 ± 0.01	34 ± 0.01	260 ± 0.01	8.2 ± 0.2

[a] β-Galactosidase activities were determined from the lysates of cells carrying transcriptional fusion constructs of promoters grown to log phase in minimal media with Fru, Glu, and Gly as indicated. Promoter fusions included the start codon and genomic region (indicated in bp) immediately upstream of each target gene. Note that *glpR-pfkB* and HvoA0327-*kdgK2* are operons most likely transcribed from these promoters. β-Galactosidase activity values increased by *glpR* mutation are in bold. Experiments were performed in biological triplicate, and the means ± SD were calculated. ND, not determined.

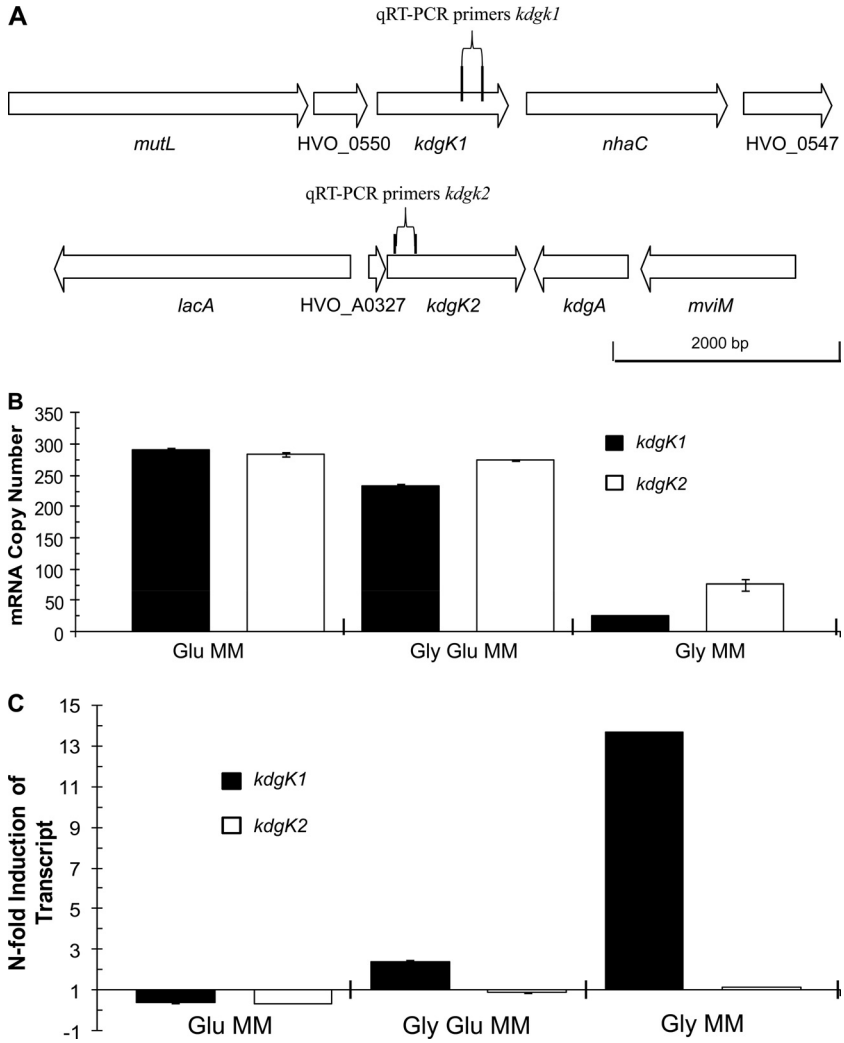

FIG. 3. Genomic organization and transcript analysis of KDGK genes located on the chromosome and megaplasmid pHV4 of *H. volcanii*. (A) Schematic representations of chromosomal *kdgK1* (upper) and related pHV4-carried *kdgK2* (lower) genes and their neighbors and locations of annealing sites (represented as vertical lines) for qRT-PCR primers. (B) Absolute quantification of transcripts specific for *kdgK1* and *kdgK2* reveals increased transcript levels for both genes during growth on glucose, regardless of glycerol supplementation. Transcripts specific for *kdgK1* and *kdgK2* were upregulated 12-fold and 4-fold, respectively, in the presence of glucose-containing media compared to the levels obtained with glycerol alone. Transcript levels were derived from qRT-PCR as described in Materials and Methods. (C) Relative quantifications of transcript levels specific for *kdgK1* and *kdgK2.* Chromosomal *kdgK1* transcripts are upregulated in a *glpR* mutant (KS8) in the presence of glycerol, regardless of glucose supplementation, compared to the level for parent H26. Transcript levels were derived by qRT-PCR. Calculations are based on the *n*-fold value for induction of transcripts in the designated minimal media for *glpR*-deficient cells compared to the level for parent H26. Results were normalized to the *n*-fold value for induction of the internal control, *ribL*. Experiments were performed in triplicate, and the means ± SD were calculated.

Furthermore, chromosomally located *kdgK1* was significantly upregulated in the *glpR* mutant compared to the level for parent H26 when cells were grown on glycerol minimal medium (14-fold) but was relatively unaltered when cells were grown on media with glucose (i.e., 0.38-fold ± 0.02-fold lower in the presence of glucose alone and 2.4-fold ± 0.14-fold higher on glucose plus glycerol) (Fig. 3C). Unlike *kdgK1*, the *glpR* mutation had little if any impact on *kdgK2* transcript levels compared to those for parent H26 on all media examined (i.e., Glu MM, Gly-Glu MM, and Gly MM) (Fig. 3C).

On the basis of the qRT-PCR finding that GlpR may serve as a repressor of *kdgK1* transcript levels during growth on glycerol, KDGK enzyme activity in both parent and *glpR* mutant strains was monitored to determine the role of GlpR at the protein level. Similar to transcript levels, KDGK activity was reduced when cells were grown on glycerol compared to the level obtained with glucose (Fig. 4). Likewise, deletion of *glpR* resulted in significant increases in KDGK activity on glycerol, with the increase on glycerol and glucose modest (2-fold) compared to the level obtained with glycerol alone (7-fold) (Fig. 4). These levels were reduced to parental levels by providing *glpR* in *trans* (Fig. 4). The *glpR* knockout did not affect KDGK activity when cells were grown with glucose as the sole carbon source (Fig. 4) or on peptide-rich YPC media with or without glucose or fructose (see Fig. S5 in the supplemental material). On the basis of these results, the KDGK activity of

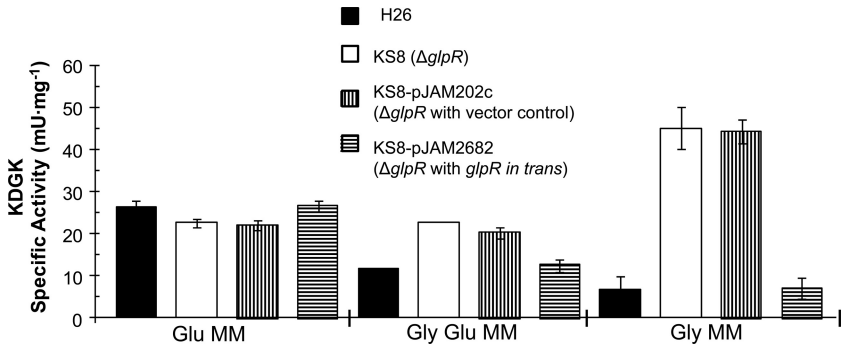

FIG. 4. KDGK activity is increased by deletion of *glpR* in cells grown on glycerol minimal medium. KDGK specific activities were determined from cell lysates of log-phase *H. volcanii* H26 (parent), KS8 (*glpR* mutant), KS8-pJAM202c (*glpR* mutant with vector control), and KS8-pJAM2682 (*glpR* mutant with *glpR* in *trans*) grown on Glu, Gly-Glu, and Gly as indicated. Experiments were performed in biological triplicate, and the means ± SD were calculated.

H. volcanii was consistent with the qRT-PCR data in which *glpR* was needed for repression of *kdgK1* transcript levels on glycerol. Furthermore, KDGK specific activity (7.1 to 45 $mU \cdot mg^{-1}$), although higher, was within a reasonable range observed for other haloarchaea (i.e., *H. saccharolyticus* at 2 to 5 $mU \cdot mg^{-1}$) (20).

To further investigate the role of GlpR at the level of KDGK transcription, the *kdgK1* and *kdgK2* promoter regions were fused to a *bgaH*-based transcriptional reporter (as described above). Reporter fusions of various lengths and start sites were generated to ensure that the entire promoter and potential regulatory elements were included for analysis, and transcription of the *kdgK* promoter constructs was monitored by an assay of β-galactosidase activity (see Materials and Methods and Table 2 for details). Although the β-galactosidase activities of the *kdgK1*- and *kdgK2-bgaH* transcriptional fusions were somewhat high on glycerol alone (Gly MM), these activities increased significantly upon glucose supplementation (Table 2). Furthermore, the β-galactosidase activities of both *kdgK1-bgaH* fusions were higher in the *glpR* mutant than in the parent when the cells were grown on glycerol alone (Table 2). In contrast to *kdgK1*, deletion of *glpR* did not affect transcription of the *kdgK2*-promoter fusions as measured using the reporter constructs (Table 2). Thus, GlpR appears to reduce KDGK activity and *kdgK1* transcript levels through transcriptional repression of *kdgK1* when cells are grown on glycerol. It should be noted that both the 89- and the 524-bp *kdgK1* and 122-bp HVO_A0327-*kdgK2* promoters are relatively robust at driving expression of the heterologous *bgaH* reporter. β-Galactosidase activity for the *kdgK* promoters was greater than that of the *H. cutirubrum* rRNA P2 promoter (Table 2), used routinely for high-level production of proteins in *H. volcanii* (22, 40).

While our data reveal that GlpR is required for the transcriptional repression of *kdgK1* and the reduction of KDGK enzyme activity when the cells are grown on glycerol, it remains to be determined why the levels of β-galactosidase activity are relatively high for both *kdgK1-bgaH* reporter fusions on glycerol compared to the nearly baseline level of transcripts detected for *kdgK1* by qRT-PCR during growth on this same medium. Posttranscriptional mechanisms and/or insufficient levels of GlpR, needed to repress transcription of the *kdgK1-bgaH* reporter fusion on the multicopy plasmids pJAM2705 and pJAM2706, may explain these findings.

GlpR and diauxic growth. The data presented here indicate that GlpR represses transcription of the *glpR-pfkB* operon and *kdgK1* in the presence of glycerol and that GlpR is no longer an active repressor of these operons when cells are grown on media with fructose or glucose. It is unclear, however, whether GlpR and/or these operons are control points for the diauxie previously observed during growth on glucose and glycerol, in which glucose metabolism is repressed by glycerol (38) (see Fig. S6 in the supplemental material). To directly examine the role of GlpR in this phenomenon and determine whether glycerol represses fructose metabolism, the *glpR* deletion was introduced into a previously described glycerol kinase (*glpK*) mutant strain, KS4 (38), for phenotypic analysis. Since both *glpR* and *glpK* deletions are in separate operons and markerless, this double mutant strain was readily generated. Parent and *glpK* mutant strains were analyzed as a replicate of previous experiments (38) along with single *glpR* and double *glpR glpK* mutant strains for growth and carbon utilization on minimal media, including fructose, glucose, and/or glycerol. Interestingly, unlike the glycerol-mediated catabolite repression of glucose metabolism observed for *H. volcanii* cells (38), glycerol and fructose are coutilized when provided at equimolar concentrations (see Fig. S7 in the supplemental material). Furthermore, GlpR did not appear to regulate the diauxic growth of *H. volcanii* on glycerol and glucose, based on the finding that the *glpR* mutation did not alleviate this catabolite repression (see Fig. S6 in the supplemental material).

The promoter regions for *kdgK1* and *glpR-pfkB* include a putative GlpR binding motif. Based on the finding that GlpR likely controls *pfkB* and *kdgK1* transcription within the 188-bp and 89-bp region upstream of the translational start codon for each gene, respectively, these regions were analyzed to determine putative promoter elements and the GlpR binding motif(s). Shine-Dalgarno sites and promoter elements, including the TFB responsive element (BRE) and the TATA box, were predicted based on consensus sequences (15). Next, inverted repeats located near these elements were aligned to identify the potential GlpR binding site(s). With this approach, an inverted hexameric repeat, $TCSnCn_{(3-4)}SSnGGA$ (where S is

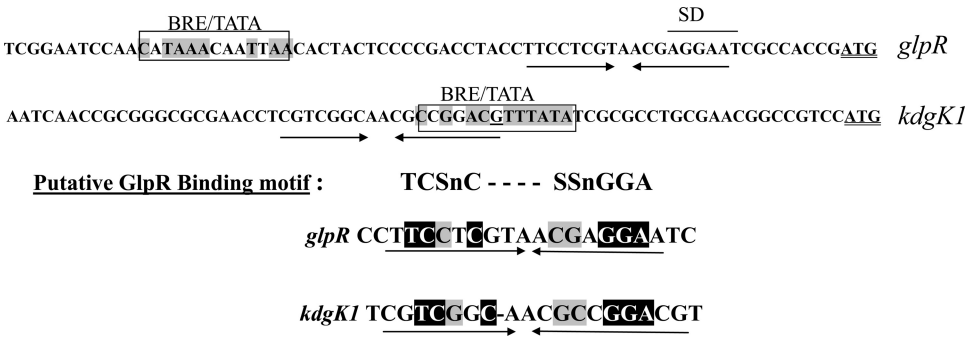

FIG. 5. Genomic regions upstream of *glpR-pfkB* and *kdgK1* include a conserved inverted repeat that may serve in GlpR binding and transcriptional repression. Regions upstream of *glpR-pfkB* and *kdgK1* were analyzed for potential GlpR binding sites, such as inverted repeats near putative Shine Dalgarno (SD) sites and promoter elements, including the TFB responsive element (BRE) and the TATA box (the consensus sequences were CRnAAT for BRE and TTTAWA for the TATA box, where W is A or T, R is A or G, and n is any nucleotide base). Residues matching the consensus BRE/TATA box sequence are boxed and highlighted in gray. SD sites are indicated by a line above the DNA sequence, and the translational start codon is double underlined. An inverted hexameric repeat with a consensus sequence, $TCSnCn_{(3-4)}SSnGGA$ (where S is C or G and n is any nucleotide base), was found common to the *glpR-pfkB* and *kdgK1* promoter regions. Inverted repeats are indicated, with arrows displaying directionality.

G or C and n is any nucleotide), which overlaps the putative *kdgK1* promoter and is downstream of the putative *glpR-pfkB* promoter, was identified (Fig. 5). This motif was not found within the *kdgK2* promoter region, consistent with our findings that GlpR regulates both *kdgK1* and *pfkB* but not *kdgK2.* Future investigation is expected to provide insight as to whether GlpR binds this motif and represses transcription from the *kdgK1* and *glpR-pfkB* promoters when cells are grown on glycerol.

Conclusions. In this study, we demonstrate that the DeoR/GlpR-type GlpR protein represses the transcription of genes encoding both fructose and glucose metabolic enzymes when *H. volcanii* cells are grown on glycerol minimal medium. The transcript levels and activities of key enzymes of fructose and glucose metabolism, PFK (*pfkB*) and KDGK (*kdgK1*), were reduced in the presence of glycerol alone compared to the levels obtained with fructose or glucose. Analysis of the transcriptional fusions of the *pfkB* and *kdgK1* promoters in a *glpR* mutant strain revealed that GlpR was required for transcriptional repression of these genes during growth on glycerol. In contrast, transcription from the *kdgK2* promoter, although reduced by glycerol, was not significantly altered by the *glpR* mutation, suggesting that an additional regulator is used for *kdgK2*. The *glpR* and *pfkB* genes were cotranscribed under a common promoter based on RT-PCR analysis, suggesting that GlpR also serves as an autoregulator, and transcription of the *glpR-pfkB* operon was reduced during growth on glycerol alone compared to that obtained with growth on fructose or glucose. The results presented here provide the first genetic and biochemical evidence of a DeoR/GlpR-type transcriptional repressor protein in haloarchaea. Future biochemical characterization of GlpR and its effector is expected to provide further insight into the transcriptional regulation of sugar metabolism in *H. volcanii* as well as other microorganisms with similar gene organizations. Further analysis will also provide new insight into how DeoR/GlpR family members can interact with and regulate archaeal basal-transcriptional machineries composed of eukaryote-like RNAP and TBP and TFB proteins.

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