Identification and Characterization of gshA, a Gene Encoding the Glutamate-Cysteine Ligase in the Halophilic Archaeon * Halofax volcanii*

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Halophilic archaea were found to contain in their cytoplasm millimolar concentrations of γ-glutamylcysteine (γGC) instead of glutathione. Previous analysis of the genome sequence of the archaeon Halobacterium sp. strain NRC-1 has indicated the presence of a sequence homologous to sequences known to encode the glutamate-cysteine ligase GshA. We report here the identification of the gshA gene in the extremely halophilic archaeon *Halofax volcanii* and show that *H. volcanii gshA* directs in vivo the synthesis and accumulation of γGC. We also show that the *H. volcanii* gene when expressed in an *Escherichia coli* strain lacking functional GshA is able to restore synthesis of glutathione.

Many organisms contain millimolar concentrations of low-molecular-weight thiol compounds that participate in a number of important biological functions involving thiol-disulfide exchanges (7). In particular, they serve to maintain an intracellular reducing environment, to provide reducing power for key reductive enzymes, to combat the effects of oxidative and disulfide stress, and to detoxify xenobiotic compounds (7). Glutathione (GSH), a cysteine-containing tripeptide, is the best-characterized low-molecular-weight thiol (7, 19, 21). GSH is made in a highly conserved two-step ATP-dependent process by two unrelated peptide bond-forming enzymes (3, 21). The γ-carboxyl group of L-glutamate and the amino group of L-cysteine are ligated by the enzyme glutamylcysteine (GC) ligase EC 6.3.2.2 (GshA, encoded by gshA), which is then condensed with glycine in a reaction catalyzed by GSH synthetase (GshB, encoded by gshB) to form GSH (10, 38). GSH is found primarily in gram-negative bacteria and eukaryocytes and only rarely in gram-positive bacteria (26). Fahey and coworkers showed that GSH is absent from the high-GC gram-positive actinomycetes which naturally produce d-threonine (11), whereas in some was the same as the HY+/T medium except that the yeast extract was replaced by 5 g of Casamino Acids (Difco). H minimal medium (HMM) contained 150 g of NaCl, 36.9 g of MgSO₄ · 7H₂O, 5 ml of a 1 M KCl solution, 1.8 ml of a 75-mg/liter MnCl₂ solution, 5 g yeast extract (Difco), and Tris-HCl (pH 7.2) at a final concentration of 50 mM. After autoclaving and cooling, 5 ml of 10% (wt/vol) CaCl₂ and 4 ml of 10% (wt/vol) sodium succinate, 2 ml of 0.5 M K₂HPO₄, and Tris-HCl (pH 7.2) at a final concentration of 50 mM. After autoclaving and cooling, the following materials were added: 5 ml of 10% (wt/vol) CaCl₂, 1 ml trace elements solution (23), 0.8 ml of 1 mg/ml thiamine, and 0.1 ml of 1 mg/ml biotin.

Gene knockouts and transformation procedures. The gene knockouts were performed according to the “pop-in/pop-out” methodology, as previously described (5). Briefly, the upstream and downstream flanking regions of the genes to be deleted are PCR amplified and cloned together into the “suicidal plasmid” pGB70, which carries the pyrE selectable genetic marker but cannot replicate autonomously in *H. volcanii*. The plasmids are transformed into an *H. volcanii* ΔpyrE mutant, and transformants in which the plasmids have been integrated.

MATERIALS AND METHODS

**Strains and culture conditions.** The properties of the *H. volcanii, E. coli, and plasmid strains used in this work are given in Table 1. *H. volcanii* was routinely grown in rich (HY) medium containing (per liter) 150 g of NaCl, 36.9 g of MgSO₄ · 7H₂O, 5 ml of a 1 M KCl solution, 1.8 ml of a 75-mg/liter MnCl₂ solution, 5 g yeast extract (Difco), and Tris-HCl (pH 7.2) at a final concentration of 50 mM. After autoclaving and cooling, 5 ml of 10% (wt/vol) CaCl₂ and 4 ml of 10% (wt/vol) sodium succinate, 2 ml of 0.5 M K₂HPO₄, and Tris-HCl (pH 7.2) at a final concentration of 50 mM. After autoclaving and cooling, the following materials were added: 5 ml of 10% (wt/vol) CaCl₂, 1 ml trace elements solution (23), 0.8 ml of 1 mg/ml thiamine, and 0.1 ml of 1 mg/ml biotin.

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RESULTS AND DISCUSSION

Identification of the Haloferax volcanii gshA gene. We have extended the previously reported computational methodology (6) to identify GshA homologs in Halobacteriaceae species. The Halobacterium sp. strain NRC-1 VNG1397C ORF sequence was first used as a query in BLAST searches of the H. volcanii DS2 genome (25). A single ORF, HVO1668, annotated as encoding a hypothetical protein, was identified. The HVO1668 protein consists of 345 amino acid residues, has an isoelectric point of 4.37, and shares 68% identity with VNG1397C. Domain analysis established that it belongs to the Pfam GCS2 glutamate-cysteine ligase family. The H. volcanii HVO1668 gshA-like gene is located between flpA (HVO1669), encoding a fibrillarin-like pre-rRNA processing protein, and trmB (HVO1667), a putative ArsR family transcriptional regulator (Fig. 1A). H. volcanii GshA shares limited sequence relatedness to E. coli GshA, about 15% identity (Fig. 1B). Homologous gshA-like genes were identified in the genomes of 10 other halophilic archaea sharing strong similarity, ranging between 64 to 75% in sequence identity (see Fig. 5).

The H. volcanii gshA-like gene is responsible for in vivo synthesis of γGC. H. volcanii (wild type) was grown in HMM (a GSH-free medium). Cell extracts were assayed for low-molecular-weight free thiols after derivatization with the thiol-specific reagent mBBr followed by separation by HPLC (26). Figure 2A shows that the two major thiols in the chromatogram are γGC and CoASH. No GSH was detected, in confirmation with previous reports for the halophilic archaeon Halobacterium halobium (30, 36). Free thiols were authenticated in control experiments in which cell extracts were treated with N-ethylmaleimide to block free thiols prior to reaction with mBBr (26). To determine whether the H. volcanii gshA-like gene is responsible for γGC synthesis, we employed the “pop-in/pop-out” strategy for constructing the gene knockout (see Materials and Methods). In this procedure, if the deletion of the target gene has no effect on the growth properties of the cells, it is expected that in about one-half of the cells the

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features</th>
<th>Source or reference</th>
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<td>ΔpyrE2 ΔleuA ΔtrpA ΔhrdA</td>
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</tr>
<tr>
<td>WR632</td>
<td>WR541 gshA</td>
<td>This work</td>
</tr>
<tr>
<td>WR633</td>
<td>WR032 (pWL-nov-gsh)</td>
<td>This work</td>
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<tr>
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<td>80lacZAM15 mcrA Δ(mnr-hsdRMS-mcrBC) araD139 Δ(ara leu)7697 Δ(lacX74 galU galK rpsL) (Str*) mphG recA1 F′ proAB – lacPZAM15</td>
<td>Invitrogen</td>
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<td>WP758 (pUC120-Gsh)</td>
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</tr>
<tr>
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<td>pUC19 containing the H. volcanii pyrE2 coding region under the H. salinarum ferredoxin promoter</td>
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<tr>
<td>pMM101</td>
<td>pUC19-H. volcanii shuttle vector containing the novobiocin resistance gene and the promoter region of the H. salinarum ferredoxin gene</td>
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<td>H. volcanii gshA flanking regions cloned into pGB70</td>
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<tr>
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</tr>
<tr>
<td>pUC120-Gsh</td>
<td>pUC120 carrying the H. volcanii gshA gene under the lacZ promoter and operator</td>
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H. volcanii strains

WR541
WR632
WR633
DH125
WP758
MM1006
pGB70
pMM101
pLGsh
pWL-Nov-Gsh
pUC120-Gsh

E. coli strains

Into the chromosome are selected on CA + T plates that lack uracil. Upon counterselection on HY + T plates containing 100 μg/ml 5-fluoroorotic acid, the only cells that survive are those in which the integrated plasmids have been excised by spontaneous intrachromosomal homologous recombination, either restoring the wild-type gene or resulting in its deletion. In order to distinguish between colonies that carry the deletion and those that reverted to the wild-type allele, colony PCR was performed on the 5-fluoroorotic acid-resistant colonies by using DNA primers whose sequences are located at the 5′ and 3′ flanking regions of the target knockout gene. Transformation of H. volcanii was carried out using the polyethylene glycol method as described previously (5).

Analysis of thiols from Haloferax volcanii. H. volcanii cultures were grown in HMM for thiol analysis. Cells were harvested and washed by centrifugation. Washed cell pellets (50 to 100 mg) were resuspended in 1 ml of 50% (vol/vol) acetonitrile in Tris-HCl buffer (20 mM, pH 8.0) containing 2 mM monobromo-bimine (mBBr; Calbiochem) and incubated at 60°C for 15 min in the dark. Control samples were treated with 5 mM N-ethylmaleimide for 10 min under the same conditions before the addition of mBBr (to give 2 mM). The cellular debris was removed by centrifugation, and the samples were diluted in 10 mM aqueous methanol sulfonic acid for reverse-phase high-pressure liquid chromatography (HPLC) analysis or for frozen storage for future analyses. Thiol standards were prepared as described previously (8).

HPLC analysis of thiol-bimane derivatives. Duplicate samples of cell extracts were routinely analyzed for thiols as their bimane derivatives (26, 29). The chromatographic conditions used in these protocols, the sources for reagents, and the preparation of thiol-bimane standards used have been described in detail elsewhere (8, 26). Briefly, we used the coenzyme A method, which constituted a tetraethylammonium phosphate (TBAP) ion-pairing protocol designed for the separation of coenzyme A-bimane derivatives (8). This method used a C8 RP column (C8 Symmetry, 3.9 by 150 mm; Waters) at a flow rate of 1.0 ml min⁻¹. The chromatographic protocol employed solvents and gradients as follows: solvent A, 1.0% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP, pH 3.4, solvent B, 90% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP. The steps were as follows: 10% solvent B at time zero, 15 min at 25% solvent A, 1.0% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP, followed by a wash, equilibration, and reinjection.

Genomic data analysis. The Haloferax volcanii genome sequence data were obtained from the UCSC Genome Browser, available at the following website: http://archaea.ucsc.edu/cgi-bin/hgGateway?db=hvolVolc1. Pairwise and multiple-amino-acid sequence alignments were prepared by using the EMBL Blast2W server (17). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 4 (37). The phylogeny of the γGC homologs was inferred using the neighbor-joining method (34). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The protein sequences used in this analysis and their database accession numbers are listed in Table S1 in the supplemental material.
FIG. 1. H. volcanii gshA-like gene, chromosomal organization, and sequence alignment. (A) The gshA gene (HVO1668) is located upstream of trmB (HVO1667), encoding a putative ArsR family transcriptional regulator, and downstream of flpA (HVO1669), encoding a fibrillarin-like pre-rRNA processing protein. (B) Multiple sequence alignment of the deduced protein sequences of the H. volcanii and Halobacterium sp. strain NRC-1 gshA-like genes and E. coli gshA.

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
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GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI

GshA_HalVol
GshA_HalNRC-1
GSH1_ECOLI
excision of the chromosomally integrated plasmid will leave behind the wild-type allele of the target gene and that in about one-half of the cells the excision will create the desired deletion. Thus, the deletion plasmid pLGsh was transformed into *H. volcanii* strain WR541 and integrated into its chromosome to create “pop-in” colonies. Following “pop-out” counterselection, it was found that in about one-half of the cells the deletion of the gshA-like gene had occurred, as determined by PCR.

FIG. 2. Representative HPLC analysis of the low-molecular-weight free thiols in wild-type *H. volcanii*, *H. volcanii* ΔgshA, and a derivative *H. volcanii* strain containing an integrated, complementing gshA copy. (A) *H. volcanii* gshA+ (WR541, wild type) cell extracts derivatized with mBBr. (B) *H. volcanii* ΔgshA (WR322) cell extracts derivatized with mBBr. (C) *H. volcanii* ΔgshA, transformed with plasmid pWL-nov-gsh (W633) cell extracts derivatized with mBBr. (D) Standards, with a known mixture of thiol-mb derivatives containing GSH, N-acetylcysteine (NAC), cysteine (Cys), CoASH, and γGC. Peaks labeled R that were found in the control samples, in which thiols had been blocked with N-ethylmaleimide prior to treatment with mBBr, are assumed to represent fluorescent components from the cells, reagent-derived components, or thiols having atypical reactivity. DTT, dithiothreitol. The HPLC analysis of cellular thiols was repeated two or three times, with no significant difference in the results.
analysis, giving strain WR632. To determine the effect of the deletion on γGC synthesis, strain WR632 was grown in HMM to an optical density at 600 nm of 1.7 and thiols were extracted and derivatized for HPLC analysis. Figure 2B shows that, in comparison with what was found in the wild-type strain, there was no detectable γGC present in the mutant strain, whereas CoASH levels were similar in both strains.

To assess whether H. volcanii gshA plays a role in managing...
oxidative stress, we challenged the wild type and the ΔgshA mutant with different oxidative stress agents. Figure 3 shows that 0.5 mM diamide, a thiol-specific oxidant (16), significantly inhibits the growth of ΔgshA mutant cells in comparison to those of the wild type. In contrast, 1.0 mM paraquat, 0.1 mM τ-butyl hydroperoxide, 0.5 mM hydrogen peroxide, and 0.01 mM menadione had a much less pronounced effect on growth (data not shown).

**Complementation of WR632 with H. volcanii HVO1668 restores parental γGC levels.** To confirm that the absence of γGC from strain WR632 was caused by the deletion of the HVO1668 sequence, the gene corresponding to HVO1668 was amplified by PCR, cloned into the haloarchaeal plasmid pMM101 to obtain pWL-nov-gsh, and transformed into WR632 to obtain WR633. WR633 cells were grown in HMM containing 0.5 μg/ml novobiocin to an optical density at 600 nm of 1.7, and the low-molecular-weight thiols were extracted and derivatized for HPLC analysis. Figure 2C shows the presence of γGC in the complemented strain. These experiments establish that the *H. volcanii* protein HVO1668 is the enzyme that synthesizes γGC. In their studies of γGC formation in *Halobacterium halobium*, Sundquist and Fahey hypothesized that halophiles might utilize γGC rather than GSH since they found that γGC is at least as stable to metal-catalyzed oxidation in high salt concentrations as GSH, which is not the case in low salt concentrations (36). It is interesting to note therefore that nonhalophiles *L. kimchi* and *L. mesenteroides* make large amounts of γGC but lack GSH (15). Further studies are required to elucidate the particular advantage(s), if at all, related to why some bacteria employ GSH in place of the simpler compound γGC. A possibly related issue concerns the manner in which the halophiles are, to date, the only *Archaea* reported to have the capacity to produce γGC, a phenomenon that might be the result of a highly specific lateral gene transfer event. Interestingly, we noted the presence in the archaeon *Methanosphaera stadtmanae* DSM 3091 of a gene encoding a 467-amino-acid protein sharing 31% sequence identity with the GshA like protein of the haloarchaeon *Natronaerobius thermophilus* JW/NM-WN-LF and 61% identity with the GshA domain of the GshF fusion protein of *Listeria monocytogenes* EGD-e.

**H. volcanii gshA complements E. coli WP758 (ΔgshA).** To determine whether *H. volcanii* gshA can substitute for *E. coli* gshA, we employed an *E. coli* strain, WP758, in which the gshA gene is deleted (32). WP758 is unable to synthesize GSH, since it lacks γGC, the substrate for GSH synthetase (GshB). *H. volcanii* gshA was amplified by PCR, cloned into the vector pUC120 to form pUC120-gsh, and expressed from the lacZ promoter. *E. coli* WP758 cells were transformed with pUC120-gsh, and colonies resistant to ampicillin were selected to produce strain MM1006. The transformants were tested for expression of the heterologous gshA gene. MM1006 cells were grown in Trypticase soy broth medium containing antibiotics, and 0.04 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at an A600 of 0.25 to induce gshA expression. Following gshA induction, complete thiol analysis was performed. The results presented in Fig. 3 show that the only free thiol detected in the nontransformed *E. coli* ΔgshA strain is CoASH; neither γGC nor GSH could be detected in the cell extracts (Fig. 4A). However, in the IPTG-induced transformed cells (MM1006), substantial amounts of GSH and CoASH and traces of γGC were detected (Fig. 4B). Surprisingly, the halophilic gshA gene is translated and properly folded in the *E. coli* milieu and functions as an active glutamate γ-cysteine ligase. Presumably, the small amount of γGC reflects its efficient conversion by the *E. coli* GshB GSH synthetase to GSH.

Although heterologous expression of haloarchaeal genes in *E. coli* has been reported (4), given their very different intracellular salt concentrations, it is by no means obvious that *H. volcanii* gshA would complement the *E. coli* ΔgshA mutant. Haloarchaeal enzymes contain a relatively high percentage of the acidic amino acid residues Asp and Glu and a relatively low content of basic residues, particularly Lys. Another striking, perhaps more important, difference is the overall lower content of hydrophobic residues in the halophilic proteins (14, 31, 39). We compared the amino acid compositions of the halophilic and *E. coli* GshA proteins. The data presented in Table 2 clearly show that the *H. volcanii* GshA protein is typical for halophilic enzymes; it has a more-than-twofold excess of negatively charged amino acid residues over positively charged ones, compared to a 1.18-fold excess of negatively charged amino acids over positively charged ones in *E. coli* GshA. Similarly, abundances of alanines and valines are also observed for halophilic GshA versus *E. coli* GshA, with averages of 17.6% versus 12.6%, respectively.

![Table 2 in the document](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4945922/figure/T2)

**Table 2. Average percents amino acid composition of *H. volcanii* and *E. coli* GshA proteins.**

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<th>Amino acid(s)</th>
<th>% Amino acid composition for GshA</th>
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* Ratio of percents amino acid composition for *H. volcanii* and *E. coli* GshA proteins.

Two controversial hypotheses have been proposed to interpret the halophilic adaptation mechanism. One mechanism argues that high-ionic-strength conditions potentially stabilize proteins through the interaction of a few key ions and large...
FIG. 5. Phylogeny of γGC ligase (GshA) homologs inferred by the neighbor-joining method (34). Phylogenetic analyses were constructed using MEGA4 (37). GshA sequences taken from halophilic archaea and those that contain GshA only are indicated by an asterisk, GshA sequences taken from bacteria that possess a GshF fusion protein (9) are indicated by a double asterisk, and sequences which show high similarity to GshA but are found in bacteria that do not possess γGC or GSH are indicated by three asterisks. Other sequences are from prokaryotes and eukaryotes that possess both GshA and GshB. Full names of organisms and accession numbers of protein sequences are given in Table S1 in the supplemental material.
water networks (22, 33). Alternatively, acidic, highly ionic surfaces and weak hydrophobic cores act as balancing factors of the endogenous capability of these proteins to maintain their fold in extreme salt concentrations (14, 33). Remarkably, despite its halophilic character, the H. volcanii gshA gene not only is well expressed in E. coli but forms an active enzyme. As has been pointed out (31, 39), the three-dimensional arrangement of the acidic residues into clusters on the protein surface may be more relevant to the stability of the halophilic proteins in low salt concentrations than the absolute numbers of acidic residues.

Phylogenetic clustering of the halophilic genes for glutamate-cysteine ligase. Figure 5 shows the phylogenetic relationship of GshA sequences from 11 halophilic archaeons with known eukaryotic and bacterial GshA proteins, including those present in species that lack GshB (e.g., L. mesenteroides) or in species containing a GshA/GshB fusion protein, for example, GshF of Listeria monocytogenes (9), GshAB of Streptococcus agalactiae (12), GshF of Haemophilus somnus (9), and the GshF-like ORF protein of Victivallis vadensis (40). In the latter two cases, the N-terminal part of the fusion proteins containing the GshA moiety was used for the alignment (see Table S1 in the supplemental material). Several features are evident. First, the halophilic GshA proteins form a distinct cluster that is well separated from other GshA proteins. Second, the M. stadtmanae DSM 3091 GshA-like sequence (24), the sole example to date of a nonhalophilic archaeon predicted to have a GshA-like protein, is far removed from the halophilic cluster, suggesting that ancestors of two archaeal families acquired genes encoding GshA proteins in an independent manner. This is supported by the fact that the M. stadtmanae DSM 3091 gshA gene has 26.7% G+C content, similar to that of the complete genome (26.7% G+C content), whereas halophilic archaeons have gshA genes with about 65% G+C content, similar to that of their genomes. Curiously, the M. stadtmanae (a human intestinal inhabitant) and the V. vadensis (an anaerobic bacterium isolated from a human fecal sample) (40) GshA-like proteins are more closely related to the L. monocytogenes, H. somnus, and S. agalactiae GshF and GshAB fusion proteins than to other bacterial GshA proteins. This unusual distribution of gshA genes among bacteria previously led us to speculate (9) that the ancestral gshF gene spread by horizontal gene transfer to other bacteria, eukaryotes, and at least one archaeon (such as Halobacterium sp. strain NRC-1), this would explain its unusual phylogenetic distribution. In this respect, it is of interest that the plant Arabidopsis thaliana γGC synthetase is structurally unrelated to mammalian, yeast, and E. coli homologs (20), which is also evident in the phylogenetic relationships shown in Fig. 4. Finally, we note that because the average G+C content of the haloarchaeal gshA genes (approximately 65%) is similar to those of other haloarchaeal genomes (64.1%), this would suggest that the putative gene transfer event took place at an early time in the evolution of the haloarchaeal archaea.

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