

Identification and Characterization of *gshA*, a Gene Encoding the Glutamate-Cysteine Ligase in the Halophilic Archaeon *Haloferax volcanii*^{∇†}

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Halophilic archaea were found to contain in their cytoplasm millimolar concentrations of γ -glutamylcysteine (γ GC) 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 *Haloferax volcanii* and show that *H. volcanii gshA* directs in vivo the synthesis and accumulation of γ GC. 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, L- γ -glutamyl-L-cysteinylglycine, 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 eukaryotes and only rarely in gram-positive bacteria (26). Fahey and coworkers showed that GSH is absent from the high-GC gram-positive actinomycetes which produce, as the major low-molecular-weight thiol, mycothiol, 1-D-*myo*-inosityl-2-(*N*-acetyl-L-cysteinyl)-amido-2-deoxy- α -D-glucopyranoside (13, 26–28, 35). GSH is also absent in *Archaea*. In *Pyrococcus furiosus*, coenzyme A SH (CoASH) is the main thiol (11), whereas in *Halobacterium salinarum*, γ GC is the predominant thiol and the organism possesses bis- γ GC reductase activity (30, 36). Similarly, *Leuconostoc kimchi* and *Leuconostoc mesenteroides*, gram-positive lactic acid bacterial

species, were recently found to contain γ GC rather than GSH (15). To date, these are the sole prokaryotic species reported to naturally produce γ GC but not GSH (6, 30). In this report, we describe the identification of the *gshA* gene in the extremely halophilic archaeon *Haloferax volcanii*. Copley and Dhillon (6) previously identified, using bioinformatic tools, an open reading frame (ORF) (gene VNG1397C) in *Halobacterium* sp. strain NRC-1 with limited sequence relatedness to known GshA proteins (6). However, no genetic or biochemical evidence was presented to substantiate their conclusion. Here, we show that *Haloferax volcanii* strain DS2 (1, 25) contains an ORF that directs in vivo the synthesis and accumulation of γ GC. We also show that the *H. volcanii* ORF, when expressed in *Escherichia coli* lacking functional GshA, is able to restore synthesis of GSH.

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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 ml of a 1 M KCl solution, 1.8 ml of a 75-mg/liter MnCl_2 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) CaCl_2 and 4 ml of thymidine (T) at 10 mg/ml were added. Agar plates contained 18 g of Bacto agar (Difco) per liter. The Casamino Acids medium (CA+T) for selection of integration of the pLGsh plasmid containing the *pyrE2* gene into the chromosome 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 (per liter) 150 g of NaCl, 36.9 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 ml of a 1 M KCl solution, 50 ml of 1 M NH_4Cl , 45 ml of 10% (vol/vol) glycerol, 5 ml of 10% (wt/vol) sodium succinate, 2 ml of 0.5 M K_2HPO_4 , 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) CaCl_2 , 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* Δ *pyrE* mutant, and transformants in which the plasmids have been integrated

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant features	Source or reference
<i>H. volcanii</i> strains		
WR541	$\Delta pyrE2 \Delta leuB \Delta trpA \Delta hrdB$	2
WR632	WR541 $\Delta gshA$	This work
WR633	WR632 (pWL-nov-gsh)	This work
<i>E. coli</i> strains		
DH12S	80d $lacZ\Delta M15 mcrA \Delta(mrr-hsdRMS-mcrBC) araD139 \Delta(ara leu)7697 \Delta(lacX74 galU galK rpsL (Str^r) nupG recA1/F' proAB^+ lacI^{\Delta} \Delta M15$	Invitrogen
WP758	<i>E. coli gshA20::Km</i>	32
MM1006	WP758 (pUC120-Gsh)	This work
Plasmids		
pGB70	pUC19 containing the <i>H. volcanii pyrE2</i> coding region under the <i>H. salinarum</i> ferredoxin promoter	5
pMM101	<i>E. coli-H. volcanii</i> shuttle vector containing the novobiocin resistance gene and the promoter region of the <i>H. salinarum</i> ferredoxin gene	18
pLGsh	<i>H. volcanii gshA</i> flanking regions cloned into pGB70	This work
pWL-Nov-Gsh	pMM101 containing the <i>H. volcanii gshA</i> gene under the ferredoxin promoter	This work
pUC120-Gsh	pUC120 carrying the <i>H. volcanii gshA</i> gene under the <i>lacZ</i> promoter and operator	This work

into the chromosome are selected on CA+T plates that lack uracil. Upon counterselection on HY+T plates containing 100 $\mu\text{g/ml}$ 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 monobromobimane (mBB; 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 mBB (to give 2 mM). The cellular debris was removed by centrifugation, and the samples were diluted in 10 mM aqueous methane 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 tetrabutylammonium 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 B, 30 min at 50% solvent B, 40 min at 75% solvent B, and 45 min at 100% solvent B, 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=haloVolc1>. Pairwise and multiple-amino-acid sequence alignments were prepared by using the EMBL ClustalW2 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.

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 fibrillar-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 archaeons 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 mBB 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 reacting with mBB (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

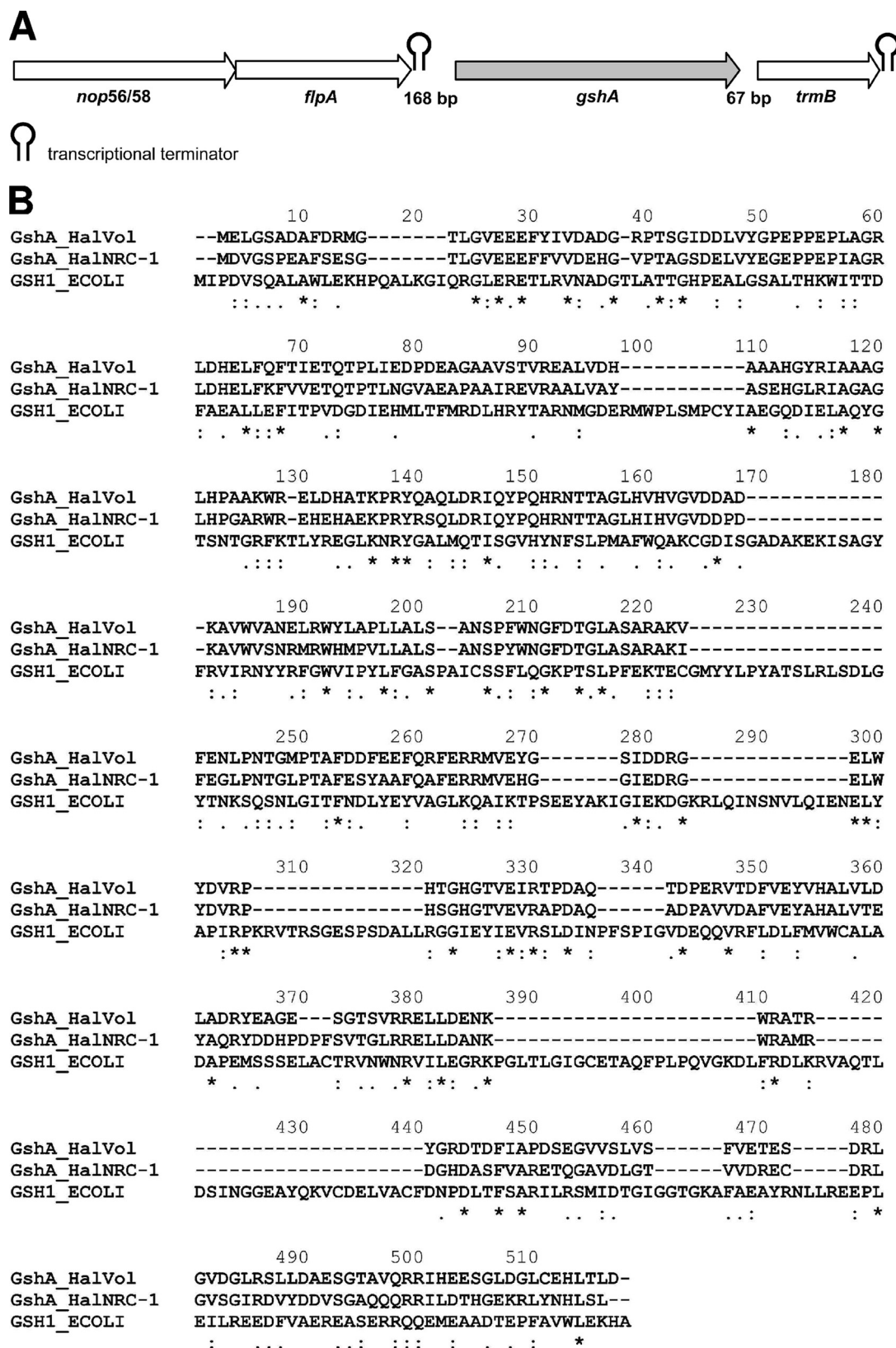


FIG. 1. *H. volcanii* *gshA*-like gene, chromosomal organization, and sequence alignment. (A) The *gshA* gene (HVO1668) is located upstream of *trmB* (HVO1667), encoding a putative ArsR family transcriptional regulator, and downstream of *flpA* (HVO1669), encoding a fibrillarin-like pre-rRNA processing 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*.

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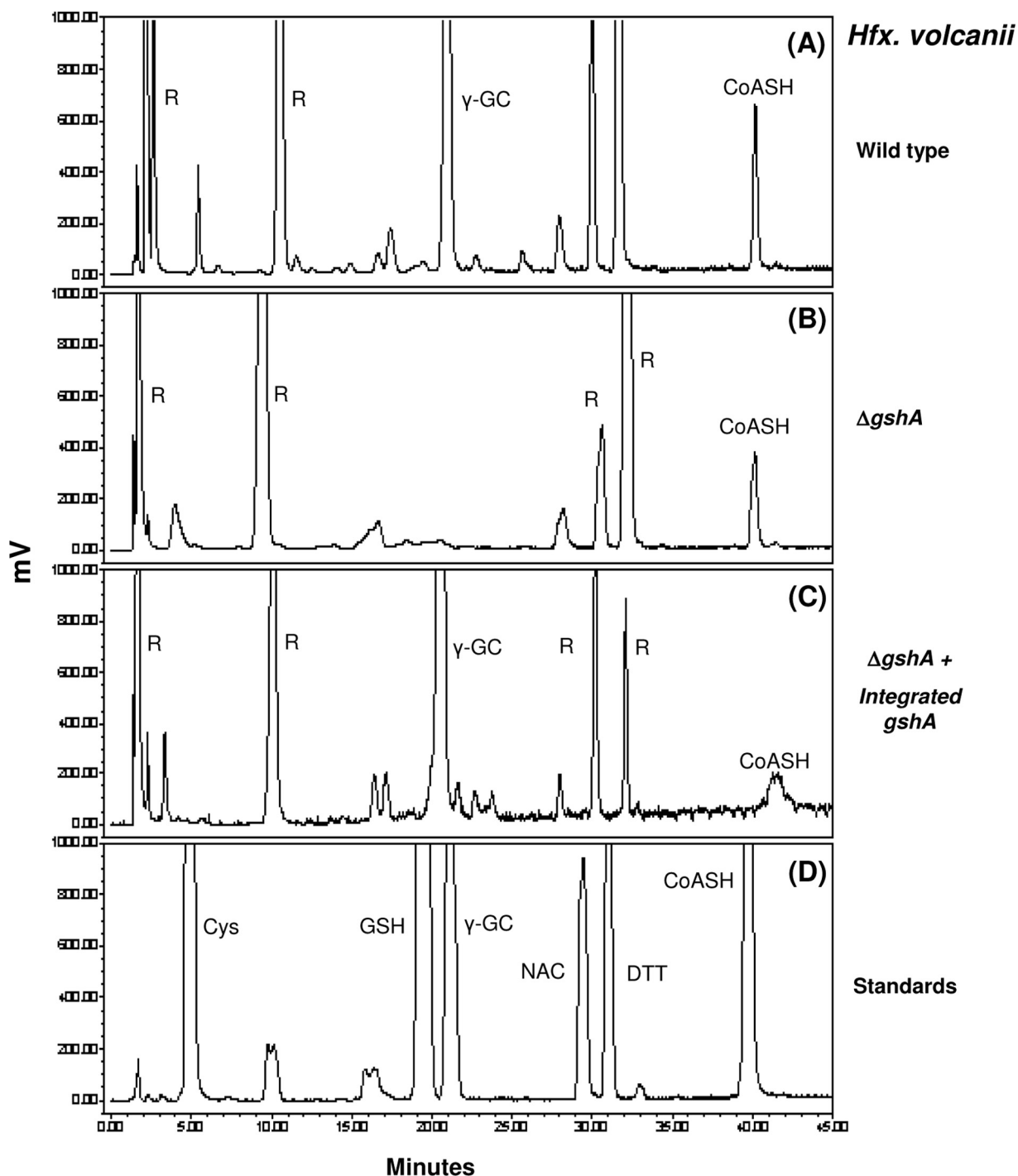


FIG. 2. Representative HPLC analysis of the low-molecular-weight free thiols in wild-type *H. volcanii*, *H. volcanii* $\Delta gshA$, and a derivative *H. volcanii* strain containing an integrated, complementing *gshA* copy. (A) *H. volcanii* *gshA*⁺ (WR541, wild type) cell extracts derivatized with mBBR. (B) *H. volcanii* $\Delta gshA$ (WR322) cell extracts derivatized with mBBR. (C) *H. volcanii* $\Delta gshA$, 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 γ GC. 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.

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

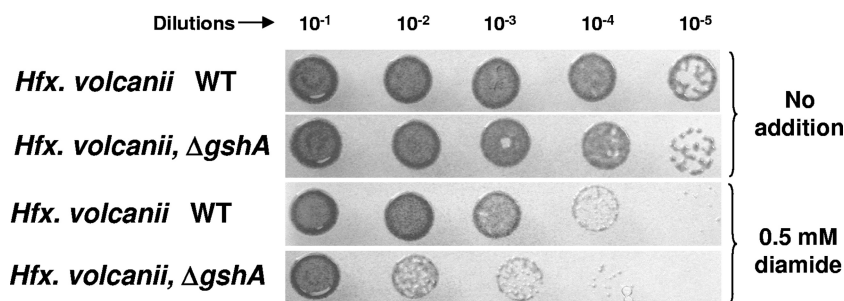


FIG. 3. Effect of oxidative agents on growth of *H. volcanii* and the $\Delta gshA$ deletion mutant. Aliquots (10 μ l) of 10-fold serial dilutions of growing cultures were spotted on HY solid medium containing 0.5 mM diamide. Plates were photographed after 4 days of incubation at 42°C. WT, wild type.

analysis, giving strain WR632. To determine the effect of the deletion on γ GC 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 γ GC present in the mutant strain, whereas CoASH levels were similar in both strains.

To assess whether *H. volcanii gshA* plays a role in managing

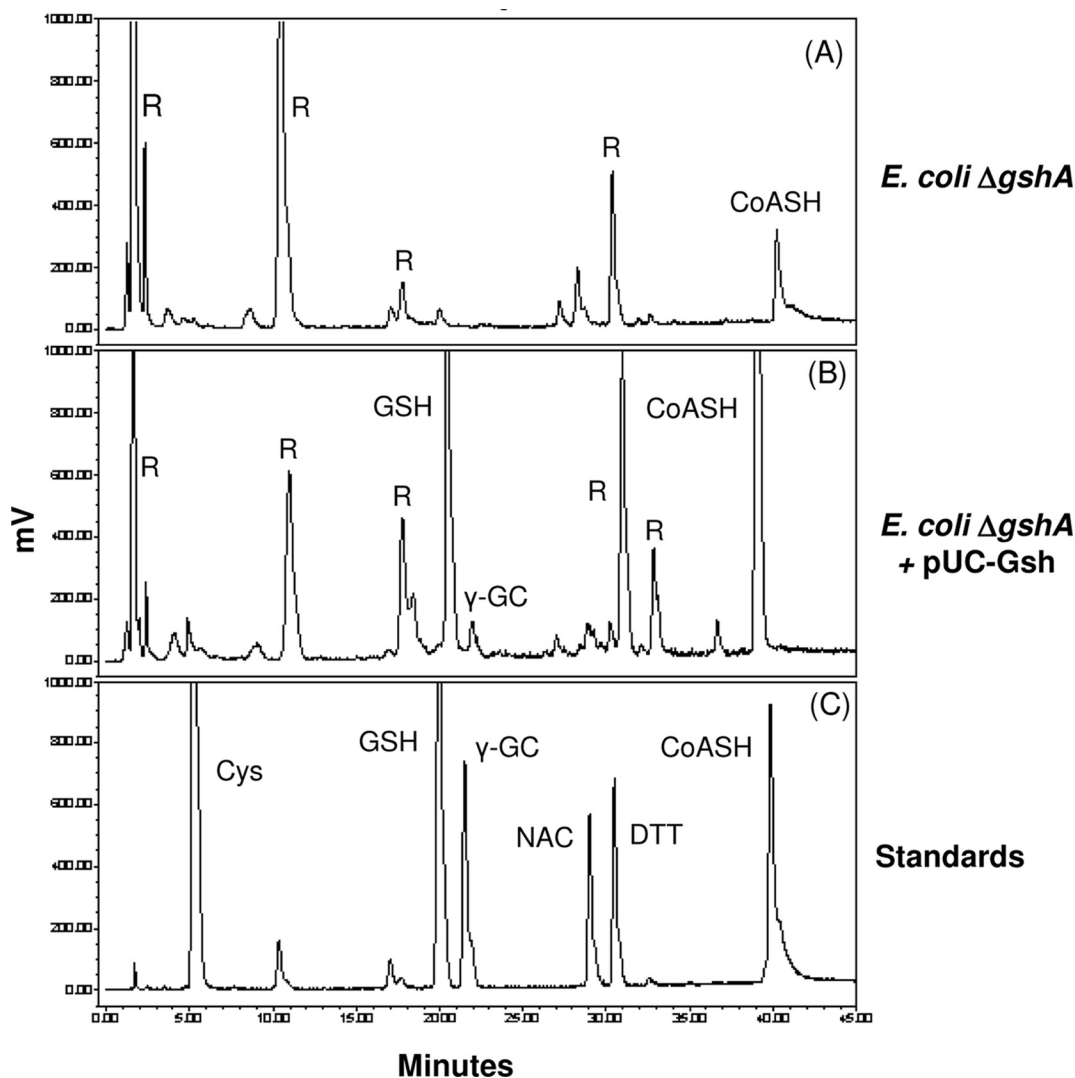


FIG. 4. Representative HPLC analysis of low-molecular-weight thiols in *E. coli* $\Delta gshA$ and in the derivative strain expressing the *H. volcanii gshA* gene. (A) *E. coli* $\Delta gshA$ (WP758) cell extracts derivatized with mBBr. (B) *E. coli* $\Delta gshA$ (pUC-Gsh) (MM1006) cells were induced with IPTG and extracts derivatized with mBBr (see text). (C) Standards, with a known mixture of thiol-mb derivatives (see legend to Fig. 2). HPLC analysis of cellular thiols was repeated two or three times, with no significant difference in the results.

oxidative stress, we challenged the wild type and the $\Delta gshA$ mutant with different oxidative stress agents. Figure 3 shows that 0.5 mM diamide, a thiol-specific oxidant (16), significantly inhibits the growth of $\Delta gshA$ 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 γ GC levels. To confirm that the absence of γ GC 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 μ g/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 γ GC in the complemented strain. These experiments establish that the *H. volcanii* protein HVO1668 is the enzyme that synthesizes γ GC. In their studies of γ GC formation in *Halobacterium halobium*, Sundquist and Fahey hypothesized that halophiles might utilize γ GC rather than GSH since they found that γ GC 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 γ GC 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 γ GC. A possibly related issue concerns the manner in which the halophiles are, to date, the only *Archaea* reported to have the capacity to produce γ GC, a phenomenon that might be the result of a highly specific lateral gene transfer event. Interestingly, we noted the presence in the archaeon *Methanospiraeta stadmanae* DSM 3091 of a gene encoding a 467-amino-acid protein sharing 31% sequence identity with the GshA like protein of the haloarchaeon *Natranaerobius 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 ($\Delta gshA$).** 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 γ GC, 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 A_{600} 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* $\Delta gshA$ strain is CoASH; neither γ GC nor GSH could be detected in the cell extracts (Fig. 4A). However, in the IPTG-induced transformed cells

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

Amino acid(s)	% Amino acid composition for GshA		
	<i>H. volcanii</i>	<i>E. coli</i>	<i>H. volcanii</i> / <i>E. coli</i> ^a
ALA	10.3	8.5	1.21
CYS	0.3	1.7	0.18
ASP	9.8	5.2	1.88
GLU	9.2	8.3	1.11
PHE	3.9	4.2	0.93
GLY	7.8	7.7	1.01
HIS	3.6	1.4	2.57
ILE	2.8	6	0.47
LYS	1.4	4.4	0.32
LEU	9.5	10.2	0.93
MET	1.1	2.5	0.44
ASN	2	3.5	0.57
PRO	5	4.8	1.04
GLN	2.5	3.9	0.64
ARG	7.8	6.6	1.18
SER	4.5	5.8	0.78
THR	6.4	5.6	1.14
VAL	7.3	4.1	1.78
TRP	1.7	1.5	1.13
TYR	3.1	4.1	0.76
ASP, GLU	19	13.5	1.41
LYS, ARG	9.2	11	0.84
ALA, VAL	17.6	12.6	1.40
PHE, TYR, TRP, ILE, LEU, MET	22.1	28.5	0.78

^a Ratio of percents amino acid composition for *H. volcanii* and *E. coli* GshA proteins.

(MM1006), substantial amounts of GSH and CoASH and traces of γ GC 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 γ GC 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* $\Delta gshA$ 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.

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 γ GC 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 γ GC 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 halophile 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% G+C content, similar to that of the complete genome (27.6% 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 in a host-related environment, since nearly all of the bacteria containing *gshF*-like genes are inhabitants of humans or domestic animals. If, as Copley and Dhillon proposed (6), GshA most probably arose in cyanobacteria (enabling protection by γ GC against reactive oxygen species) and was subsequently recruited 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* γ GC 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 halophilic archaea.

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