University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

Promoter Methylation Profiling of Nutrient Assimilation (*SulP*) and Detoxification (*ArsB*) Genes in *Galdieria SulPhuraria* from Taftan Volcanic Springs

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ARTICLE INFO

Article type:
Research Article

Article history:
Received:1 November 2025
Revised:2 December 2025
Accepted:5 December 2025

Keywords:
DNA methylation,
Epigenetic,
Adaptation,
Extremophiles,
Horizontal gene transfer.

A B S T R A C T

Poly-extreme environments, such as the Taftan volcanic complex in Iran, pose unique survival challenges for eukaryotes, requiring precise genomic regulation to balance nutrient acquisition with heavy metal detoxification. While Horizontal Gene Transfer (HGT) provides the genetic toolkit for adaptation in the red microalga Galdieria sulphuraria, the epigenetic mechanisms governing these acquired genes compared to native loci remain largely unexplored. This study investigates the role of DNA methylation in this adaptation using a "Field vs. Laboratory" de-adaptation model. We analyzed promoter methylation profiles of two functionally contrasting genes in G. sulphuraria, the native high-affinity sulfate permease (SulP) and the horizontally acquired arsenite efflux transporter (ArsB), using bisulfite conversion and targeted Sanger sequencing. Our results reveal a striking epigenetic dichotomy between metabolic and detoxification pathways. The native SulP promoter exhibited robust hypermethylation (72.1%) in field-adapted populations, which significantly decreased to 23.2% ($p < 0.0001$) under laboratory conditions. This suggests that methylation acts as a dynamic switch to repress sulfate uptake in sulfur-saturated volcanic waters, preventing intracellular toxicity. In contrast, the bacterial-origin ArsB gene maintained a stable, intermediate methylation level (~55–60%) regardless of environmental conditions ($p > 0.05$). This stability indicates a strategy of constitutive readiness, ensuring immediate defense against unpredictable arsenic fluctuations. We conclude that G. sulphuraria employs a dual epigenetic strategy: dynamic methylation remodeling for metabolic homeostasis of native genes, and static epigenetic maintenance for essential, HGT-derived detoxification systems.

Introduction

Geothermal environments represent some of the most challenging habitats on Earth, characterized by extremes in temperature, pH, and chemical composition. The Taftan volcanic complex in southeastern Iran is a prime example of such a poly-extreme environment, hosting hot springs that are not only acidic and thermal but also saturated with sulfur compounds and heavy metals (Shakeri et al., 2008). Survival in these habitats requires organisms to possess robust physiological mechanisms to maintain intracellular homeostasis against a backdrop of external toxicity. While

DOI: https://doi.org/ 10.22111/JEP.2026.54439.1103

How to Cite: Khoshkish, M., Lagzian, M. (2025). Promoter Methylation Profiling of Nutrient Assimilation (SulP) and Detoxification (ArsB) Genes in Galdieria SulPhuraria from Taftan Volcanic Springs. *Journal of Epigenetics*, 4(1), 86-95. https://doi.org/ 10.22111/JEP.2026.54439.1103

prokaryotic life in these springs has been well-documented, the eukaryotic inhabitants face unique challenges in regulating their genomic machinery to handle such abiotic stress.

Among eukaryotic extremophiles, the red microalga *Galdieria SulPhuraria* (Cyanidiales) stands out as a dominant species in acidic, sulfur-rich hot springs worldwide (Gross et al., 2001). *G. SulPhuraria* is a metabolic versatility specialist, capable of surviving pH levels as low as 0 and temperatures up to 56°C (Rossoni, Schi Nknecht, et al., 2019). A key factor in its evolutionary success is its ability to acquire essential survival genes from bacteria through Horizontal Gene Transfer (HGT), a rare phenomenon in eukaryotes (Schonknecht et al., 2013). These acquired genes often encode transporters and enzymes that allow the alga to detoxify heavy metals or metabolize unusual energy sources. However, the presence of these genes raises a critical question: how does a eukaryotic cell regulate the expression of both native and "foreign" genes to balance nutrient acquisition with toxicity avoidance?

This balance is particularly critical for sulfur metabolism and heavy metal detoxification. The sulfur-rich waters of Taftan springs provide an abundance of sulfate, a necessary nutrient, but excessive uptake can be lethal. Consequently, the expression of high-affinity sulfate permease (*SulP*) must be tightly controlled. Simultaneously, the inevitable presence of toxic metalloids like arsenic requires the active expression of detoxification pumps, such as the arsenite efflux transporter (*ArsB*), a gene of bacterial origin horizontally transferred into the *Galdieria* genome (Rossoni, Price, et al., 2019; Schonknecht et al., 2013). Regulating these opposing needs, restricting the influx of sulfur while promoting the efflux of arsenic, requires a dynamic and precise transcriptional control mechanism.

Epigenetic modification, specifically cytosine methylation (5-methylcytosine, m5C), is a primary mechanism for gene regulation in eukaryotes (Kumar et al., 2018). In contrast to the adenine methylation commonly observed in bacteria, eukaryotes primarily rely on promoter cytosine methylation to repress transcription, whereas demethylation is generally associated with transcriptional activation. (Feng et al., 2010). In plants and algae, methylation patterns in promoter regions can serve as an epigenetic switch, allowing the organism to rapidly adapt to environmental stressors without altering its underlying genetic sequence (Ferrari et al., 2023). While the genomic sequence of *G. SulPhuraria* is known, the epigenetic landscape of its stress-response genes, particularly in wild populations isolated from active volcanic sites, remains largely unexplored.

This study aims to investigate the role of DNA methylation in the environmental adaptation of *G. SulPhuraria* isolated specifically from the sulfur springs of the Taftan volcanic area in Iran. We focus on profiling the promoter methylation status of two functionally contrasting genes: the native sulfate permease (*SulP*) and the horizontally acquired arsenite resistance gene (*ArsB*). By employing bisulfite conversion coupled with targeted Sanger sequencing, we seek to determine if differential methylation serves as a regulatory mechanism to optimize survival in this poly-extreme environment. Understanding these epigenetic patterns provides new insights into how eukaryotic extremophiles "domesticate" foreign genes and fine-tune their metabolism to thrive in toxic volcanic ecosystems.

Method

Sampling Site and Sample Collection

Field sampling was conducted at a geothermal sulfur spring located on the slopes of the semi-active Taftan Volcano in Sistan and Baluchestan Province, southeastern Iran. The specific sampling site was identified near the Sobah shelter at an altitude of approximately 3,941 m above sea level (GPS coordinates: 28.59973° N, 61.110824° E). The site is characterized by significant elemental sulfur deposition (yellow crusts) and acidic thermal water. Water and sediment samples, including submerged biofilm scrapings from rock surfaces, were collected using sterile 50 mL Falcon tubes. Temperature and pH were measured in situ using a portable multi-parameter probe. Samples were stored at 4°C during transport to the laboratory.

Experimental Design and Strain Isolation

To evaluate environmentally driven epigenetic alterations, a "Field vs. Laboratory" de-adaptation model was employed. This design allows for the comparison of the *in-situ* methylation profile (stress-adapted) against a laboratory-acclimated baseline (de-adapted).

Field Sample Preservation (Stress-Adapted): A portion of the original biomass collected from the

Taftan sulfur spring was immediately preserved at the sampling site to fix the native epigenetic landscape. This sample represents the organism under poly-extreme environmental pressure.

Laboratory Control Isolation (De-adapted): To generate a biological baseline, an acid-selective enrichment strategy was employed on the remaining field sample. Approximately 1 mL of the sample was inoculated into 50 mL of modified Allen's medium adjusted to pH 2.0 using H_2SO_4. The medium contained $(NH_4)_2SO_4$ as the nitrogen source and was supplemented with trace elements. Cultures were incubated at 42°C under continuous illumination with orbital shaking at 150 rpm. This poly-extreme condition selectively promoted the growth of *G. SulPhuraria* while inhibiting neutrophilic contaminants. After 14 days, pure colonies were obtained by streaking onto solid Allen's medium plates (1.5% gellan gum, pH 2.0). The isolate was subsequently sub-cultured weekly for six weeks (approx. 40 generations) in this standard, non-toxic environment to "reset" transient environmental methylation marks.

Genomic DNA Extraction

Total genomic DNA was extracted from two distinct sources: (1) the preserved Field Biomass and (2) the log-phase Laboratory Control culture. Extraction was performed using a modified Cetyltrimethylammonium bromide (CTAB) protocol specifically optimized to overcome the high concentrations of elemental sulfur and polyphenolic inhibitors present in the volcanic biofilm. Briefly, 50 mL of culture (or approximately 200 mg of preserved field biomass) was harvested by centrifugation (3000 × g, 5 min). The cell pellet was disrupted mechanically using a bead beater (3 cycles of 30 s) with glass beads to lyse the rigid algal cell walls. The lysate was incubated in 2% CTAB extraction buffer at 65°C for 1 hour. To eliminate co-precipitating contaminants, particularly the elemental sulfur crusts ubiquitous in the field samples, two rounds of purification were performed using chloroform:isoamyl alcohol (24:1). This step was critical for partitioning sulfur crystals into the organic phase, preventing downstream inhibition of bisulfite conversion. DNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in TE buffer. DNA concentration and purity were assessed using a spectrophotometery method (A_{260}/A_{280} > 1.8) and agarose gel electrophoresis to ensure integrity prior to bisulfite treatment.

Bisulfite Conversion

To discriminate between methylated (5-mC) and unmethylated cytosines, genomic DNA was subjected to sodium bisulfite conversion using the EpiTect Bisulfite Kit (QIAGEN), following the manufacturer's instructions. Approximately 500 ng of high-quality genomic DNA was used for the reaction. During this process, unmethylated cytosine residues were deaminated to uracil, while

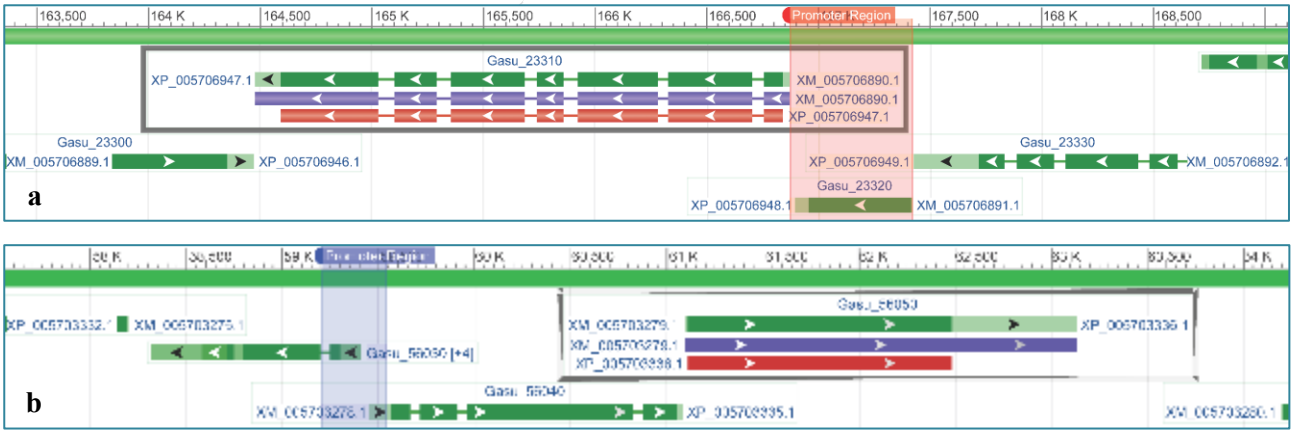

Fig. 1- Genomic architecture and target regulatory regions for *SulP* and *ArsB* in *Galdieria SulPhuraria*. (a) Organization of the high-affinity sulfate transporter locus (*SulP*). The transporter gene (Gasu_23310) is located on the negative strand, separated from the upstream 50S ribosomal protein L6 gene (Gasu_23320) by a minimal spacer (<30 bp). The shaded region highlights the targeted intragenic regulatory element, encompassing the 3' coding sequence (CDS) of Gasu_23320 and the intergenic junction, which is hypothesized to contain the transcriptional start site for *SulP*. (b) Organization of the arsenite resistance locus (*ArsB*). The arsenite efflux pump gene (Gasu_56050) is situated downstream of Gasu_56040 with a very short (~9 bp) intergenic distance, indicative of a prokaryotic-derived operon structure. The shaded region marks the distal operon promoter located upstream of Gasu_56040, which was selected for methylation profiling as the primary regulatory switch for the gene cluster. Green arrows indicate the direction of transcription; coordinates refer to scaffold NW_005178417.1.

5-methylcytosines remained unchanged. The thermal profile for conversion consisted of 98°C for 10 min followed by 64°C for 2.5 hours. The converted DNA was de*SulP*honated, purified using spin columns, and eluted in 10 µL of elution buffer. The eluted DNA was immediately used for PCR amplification or stored at -20°C.

Primer Design and In Silico Validation

Target Locus Characterization and Promoter Definition

To investigate the epigenetic regulation of nutrient assimilation and heavy metal detoxification, two distinct genomic loci were characterized on the NW_005178417.1 scaffold. Putative promoter regions were identified by analyzing upstream sequences using the Softberry TSSPlant algorithm (Shahmuradov et al., 2017), a probabilistic tool optimized for identifying Plant/Eukaryotic promoter elements (TATA boxes and Transcription Start Sites).

Sulfate Transporter: The gene encoding the high-affinity sulfate permease (*Gasu_23310*, *SulP* family) is located in a dense gene cluster on the negative strand, separated from the upstream 50S ribosomal protein L6 gene (*Gasu_23320*) by a minimal spacer of 29 bp (Fig. 1a). Given this compact organization, it hypothesized that the regulatory elements are embedded within the coding region of the upstream gene. Softberry TSSPlant analysis of this locus confirmed the presence of high-scoring TATA-box and TSS motifs located within the 3' coding sequence (CDS) of *Gasu_23320*. Consequently, the methylation profiling target was defined as a 694 bp contiguous sequence spanning the 3' terminus of the *Gasu_23320* CDS and the intergenic junction (targeting the region around 166866–167560 bp on the corresponding scaffold) (Fig. 1a).

Arsenite Efflux Protein: For the detoxification pathway, the arsenite transporter gene (*ArsB*, *Gasu_56050*) was analyzed. Genomic context analysis revealed that *Gasu_56050* is located only 9 bp downstream of the preceding gene (*Gasu_56040*). Given the prokaryotic origin of *ArsB* and the extremely short intergenic distance to the upstream gene, *ArsB* is likely co-transcribed as part of an operon-like structure and may not possess an independent promoter. Therefore, the predicted promoter region upstream of the adjacent gene (*Gasu_56040*) was considered the regulatory region controlling *ArsB* expression. Accordingly, primers were designed to target the putative promoter region upstream of the *Gasu_56040* start codon (targeting the region approx. 59174–59533 bp on the corresponding scaffold), ensuring the capture of the primary regulatory switch for this operon via a 359 bp amplicon (Fig. 1b).

Bisulfite Primer Design Strategy

Primers were designed to target these computationally defined regulatory regions using MethPrimer 2.0 (Li & Dahiya, 2002). To ensure unbiased amplification of both methylated and unmethylated alleles, the following stringent design criteria were applied: 1) Primer binding sites were restricted to regions devoid of CpG dinucleotides to prevent methylation-dependent annealing bias. 2) All non-CpG cytosines in the target sequence were computationally converted to thymines (sense strand) or adenines (antisense strand) to mimic the bisulfite conversion. 3) Primers were designed with extended lengths (25–32 bp) to compensate for the reduced complexity of the A/T-rich bisulfite-converted template, targeting a melting temperature (Tm) of 55–60°C. Based on these criteria, the specific assays were generated as table 1.

Table 1- Sequences and physicochemical properties of bisulfite-specific PCR primers used for *SulP* and *ArsB* promoter profiling. Bold and underlined nucleotides represent non-CpG cytosines converted to thymines or guanines converted to adenines to ensure specific annealing to the bisulfite-modified template.

Locus	5'- Primer Sequence - 3'	Tm °C	Amplicon	CpG sites
SulP	CTATCCAATCTAACATAAACACAACC	51,2	694	25
	TTGATTATTGTAAAGTATTTTTTT	57.4		
ArsB	TGTTATTTAAAATTTTTATTTAAGATTTTT	55.1	359	8
	TAACTCCATAAAAAAAATCACTAATTTCTT	58.7		

In Silico Specificity Verification

The specificity of the designed primer pairs was verified against the *G. SulPhuraria* genome assembly. A "bisulfite-converted genome" was generated *in silico* by substituting all cytosine

residues with thymine. The candidate primer sequences were aligned against this modified reference using BLASTn. This step confirmed that the primers uniquely targeted the intragenic regulatory region of the sulfate transporter and the upstream operon promoter of the arsenite pump, with no significant off-target amplification predicted.

Bisulfite PCR, Sequencing, and Analysis

The computationally defined promoter regions were amplified from bisulfite-converted DNA using a Hot-Start PCR protocol optimized for A/T-rich templates. After that, amplicons were gel extracted and subjected to direct Sanger sequencing using the forward PCR primers. Chromatograms were analyzed using CLC Genomics Workbench 25. Bisulfite conversion efficiency was verified by the complete conversion of non-CpG cytosines. Methylation levels at specific CpG sites were quantified by calculating the peak height ratio of Cytosine (C) to Thymine (T) according to the formula: %Methylation = [HC / (HC + HT)] ×100. This yielded the average methylation percentage for the cell population at each analyzed CpG dinucleotide.

Results

Assessment of Bisulfite Conversion Efficiency and PCR Specificity

Prior to comparative methylation profiling, the specificity of the amplification and the reliability of the bisulfite modification were strictly validated. First, PCR amplification of the A/T-rich bisulfite templates yielded single, specific bands of the expected sizes for both the *SulP* intragenic region (694 bp) and the *ArsB* operon promoter (359 bp) (Fig. 2a). This confirmed the high specificity of the primer design and the absence of non-specific byproducts or primer dimers. Subsequently, direct sequencing of these products confirmed the successful deamination of unmethylated cytosines across both assayed loci. As shown in Fig. 2b (*SulP*) and Fig. 2c (*ArsB*), alignment of the bisulfite-treated sequences against their respective untreated genomic references revealed that virtually all cytosines within non-CpG contexts (e.g., CHH and CHG) were converted to thymines. This complete conversion (>99%) ensures that any remaining cytosine peaks detected at CpG sites in subsequent analyses represent authentic methylation signals rather than incomplete chemical treatment.

Differential Methylation of the Native Sulfate Transporter (SulP)

To determine if environmental conditions influence the epigenetic regulation of sulfur transport, we analyzed the DNA methylation profiles of the native *Sulfate Transporter* (*SulP*) gene promoter. Bisulfite sequencing was performed across a genomic region containing 25 distinct CpG sites.

Visual inspection of the methylation maps revealed a striking contrast between the two groups. The laboratory-grown samples exhibited a generally hypomethylated state (Fig. 3a), characterized by a low frequency of methylated cytosines across the assayed region. The overall methylation level for the Lab sample was calculated at 23.2%.

In contrast, the Field samples displayed a robust hypermethylated profile (Fig. 3b). The majority of the 25 CpG sites in the Field sample showed high methylation ratios (represented by predominantly black pie charts), resulting in a substantial increase in overall methylation to 72.1%.

Quantitative analysis confirmed that these differences were statistically significant. A comparison of the mean percent methylation revealed that Field samples possessed significantly higher methylation levels compared to their laboratory counterparts (Fig. 3c; t-test, $p < 0.0001$). This dramatic shift suggests that environmental factors present in the field may drive epigenetic repression or modulation of the *SulP* gene.

Epigenetic Stability of the Horizontally Acquired Arsenite Pump (ArsB)

To further assess the specificity of epigenetic alterations in response to environmental conditions, we analyzed the DNA methylation profile of the *ArsB* gene. Unlike the native sulfate transporter, the *ArsB* gene displayed a high degree of epigenetic stability between the two experimental groups. Bisulfite sequencing across the target promoter region identified 8 distinct CpG sites. Visual analysis of the lollipop methylation maps (Figure 4a, b) revealed very similar methylation patterns for both the Lab and Field samples.

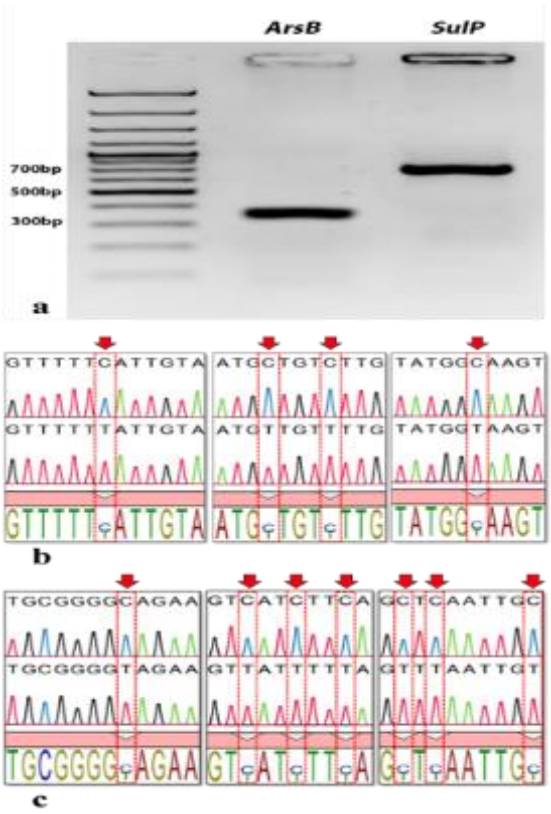

Fig. 2- Technical validation of bisulfite primer specificity and conversion efficiency. (a) Agarose gel electrophoresis of bisulfite-PCR products. Lane 1: DNA molecular weight marker; Lane 2: *ArsB* operon promoter amplicon showing a specific single band at 359 bp; Lane 2: *SulP* intragenic amplicon showing a specific single band at 694. (b, c) Comparative sequence alignments verifying bisulfite conversion efficiency for the *SulP* locus (b) and the *ArsB* locus (c). In both panels, the top sequence represents the untreated genomic reference, while the bottom sequence displays the corresponding bisulfite-treated chromatogram. Dashed boxes and arrows highlight non-CpG cytosine residues (C) in the reference that have been successfully converted to thymines (T) in the bisulfite-treated samples, confirming a conversion efficiency of >99% across both target regions.

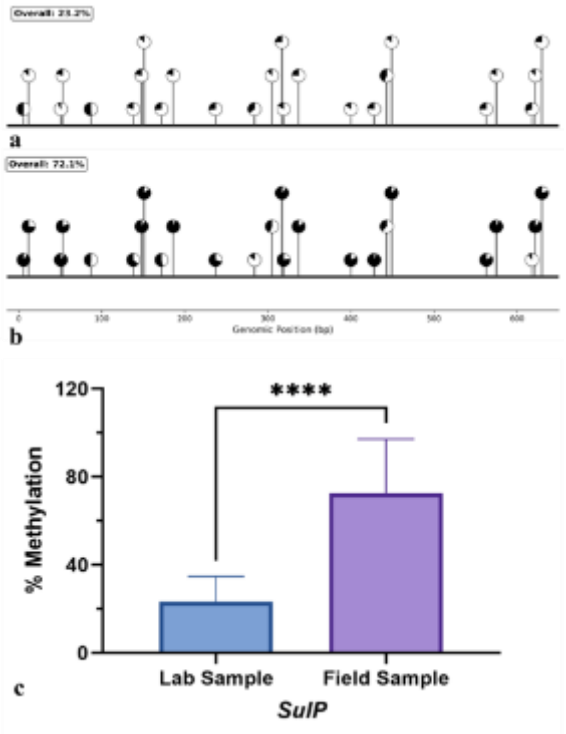

Fig. 3- Differential DNA methylation patterns of the native *Sulfate Transporter* (*SulP*) gene promoter region. (a) Lollipop methylation map illustrating the epigenetic status of 25 CpG sites within the *SulP* gene for the Lab Sample. Each vertical line represents a single CpG site mapped to its genomic position (bp). The circle at the top of each "stick" is a pie chart representing the methylation ratio at that specific site (white = unmethylated; black = methylated). The Lab sample showed a generally open chromatin state with an overall methylation average of 23.2%. (b) Corresponding lollipop map for the Field Sample. These samples exhibited widespread hypermethylation across the same genomic loci, with an overall methylation average of 72.1%. (c) Statistical comparison of the mean percent methylation between Lab and Field samples. The bar graph demonstrates a significant increase in methylation in the Field samples. Error bars represent the standard deviation. Statistical significance was determined using an unpaired t-test ($p < 0.0001$).

The pie charts at individual loci showed comparable ratios of methylated (black) to unmethylated (white) cytosines across the assayed region. Consequently, the overall methylation levels were comparable, calculated at **55.4%** for the Lab sample and **60.7%** for the Field sample.

Statistical analysis supported these observations. A comparison of the mean percent methylation demonstrated that the slight increase observed in the Field samples was not statistically meaningful (Figure 4c; t-test, $p > 0.05$). The presence of the "ns" (not significant) marker indicates that, unlike the *SulP* gene, the *ArsB* gene maintains a relatively constitutive methylation profile regardless of the environmental differences between the laboratory and field conditions.

Discussion

The survival of eukaryotic life in poly-extreme environments, such as the Taftan volcanic springs, requires a precise balance between nutrient acquisition and toxicity avoidance. While the genomic basis of adaptation in Galdieria *SulP*huraria, including horizontal gene transfer (HGT), is well-documented, the epigenetic mechanisms that regulate these diverse genetic elements remain largely unexplored. In this study, we demonstrate that DNA methylation serves as a highly specific, gene-dependent regulatory layer that facilitates adaptation to the extreme conditions of the Taftan sulfur springs. Our findings reveal a dichotomy in epigenetic control: the native sulfate transporter (*SulP*) undergoes dynamic environmental remodeling, while the horizontally acquired arsenite efflux pump (*ArsB*) maintains a rigid, constitutive methylation profile.

Differential Methylation of *SulP*: An Epigenetic Switch for Metabolic Homeostasis

The most striking finding of this study is the dramatic hypermethylation of the *SulP* promoter in field samples (72.1%) compared to laboratory cultures (23.2%), a difference that is highly statistically significant ($p < 0.0001$). This pronounced methylation shift strongly suggests that *G. SulPhuraria* utilizes DNA methylation as a dynamic regulatory mechanism to control high-affinity sulfate transport in response to environmental sulfate availability (Kumar & Mohapatra, 2021). The Taftan springs are characterized by saturation with sulfur compounds and elemental sulfur deposition, creating conditions where unrestricted sulfate uptake would likely lead to intracellular toxicity or represent an energetically wasteful expenditure of cellular resources. By hypermethylating the *SulP* promoter in its natural habitat, the alga effectively "locks" the gene into a repressed transcriptional state, thereby restricting sulfate influx and maintaining cellular sulfur homeostasis.

Upon transfer to laboratory conditions, where the organism undergoes de-adaptation, methylation levels at the *SulP* locus are dramatically reduced (23.2%), paralleling a transition to a permissive chromatin state. This epigenetic relaxation likely facilitates transcriptional activation and efficient nutrient assimilation under controlled, non-toxic growth conditions (Lamke & Baurle, 2017). This pattern aligns with established mechanisms of DNA methylation-dependent stress memory in plants and algae, wherein stress-induced changes in promoter methylation serve as reversible epigenetic switches that allow rapid adaptation to environmental fluctuations without alterations to underlying DNA sequence (Ferrari et al., 2023). The fact that this methylation remodeling is reversible upon de-adaptation, occurring within approximately six weeks of laboratory subculture, suggests that the epigenetic landscape remains plastic and responsive to environmental cues, rather than being "locked" into a permanent stress-adapted state (Crisp et al., 2016).

The regulatory pattern observed in *SulP* parallels similar mechanisms documented in plant sulfate transporters. In Arabidopsis thaliana, sulfate transporter genes (particularly SULTR1;1 and SULTR1;2) are dynamically regulated by sulfur nutritional status, with hypomethylation and transcriptional induction occurring under sulfate-limiting conditions (Kumar & Mohapatra, 2021; Zhang et al., 2014). Conversely, under sulfate-sufficient conditions, these genes are suppressed, likely through methylation-mediated repression. The inverse relationship between promoter methylation and transcriptional activity observed in the Arabidopsis sulfate assimilation pathway provides a compelling parallel to the methylation patterns documented in this study, suggesting that promoter methylation serves as a conserved epigenetic mechanism for controlling high-affinity nutrient transporters across diverse eukaryotic lineages (Meng et al., 2015).

Epigenetic Stability of *ArsB*: Constitutive Readiness in a Unpredictable Toxic Environment

In contrast to the environmentally responsive *SulP* gene, the *ArsB* gene exhibited no statistically significant difference in methylation between field and laboratory conditions, maintaining an intermediate methylation level of approximately 55–60% in both groups ($p > 0.05$). This lack of epigenetic plasticity is particularly intriguing given the bacterial origin of *ArsB*. Unlike the variable sulfate concentrations in the Taftan springs, which can be modulated through nutrient uptake and metabolism, the presence and concentration of toxic arsenic is largely unpredictable and externally imposed. The organism cannot afford the regulatory lag time associated with chromatin remodeling from a fully repressed state during

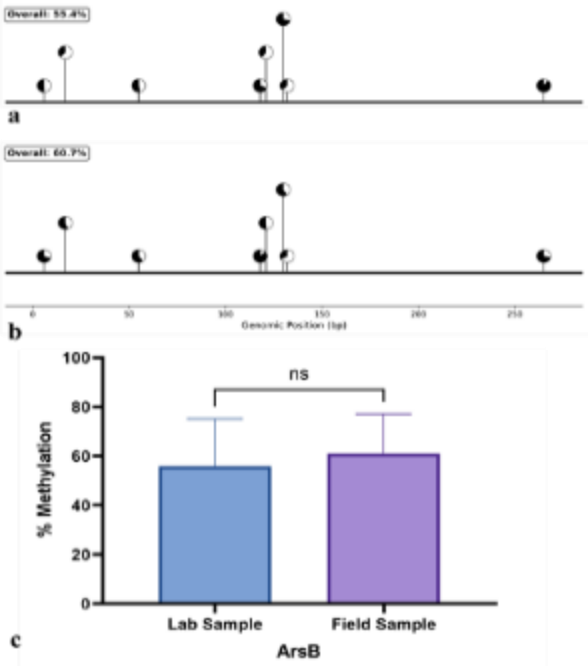

Fig. 4- Comparative DNA methylation analysis of the *ArsB* gene. (a) Lollipop methylation map detailing the epigenetic status of 8 CpG sites within the *ArsB* gene for the Lab Sample. The diagram displays the genomic position (bp) on the x-axis, with each "stick" representing a CpG site. The pie charts indicate the methylation ratio at each site (white = unmethylated; black = methylated), resulting in an overall methylation average of 55.4%. (b) Corresponding lollipop map for the Field Sample, showing a similar distribution of methylation across the same 8 loci with an overall average of 60.7%. (c) Statistical comparison of the mean percent methylation between Lab and Field samples. The bar graph indicates that the difference in methylation levels between the two groups is not statistically significant (ns), suggesting that *ArsB* methylation is stable across these environmental conditions. Error bars represent the standard deviation.

acute arsenic exposure. Therefore, *ArsB* appears to be maintained in a state of constitutive readiness, wherein the gene preserves an intermediate methylation profile that facilitates a baseline level of arsenite efflux activity at all times (Schonknecht et al., 2013). This strategy ensures that the cell maintains protective capacity against sudden or fluctuating arsenic concentrations, even at the metabolic cost of continuous detoxification pump activity.

The intermediate methylation state (~55–60%) of *ArsB* represents neither full transcriptional repression nor maximal activation, instead corresponding to a regulatory "middle ground" that maintains continuous but measured expression (Elliott et al., 2015). Recent epigenetic studies have revealed that intermediate DNA methylation states are conserved epigenetic signatures associated with intermediate levels of transcriptional activity and chromatin accessibility (Elliott et al., 2015). Exonic and promoter regions with intermediate methylation (~57% average) consistently display intermediate active chromatin marks (H3K4me1, H3K4me3) and correspond to genes with intermediate transcriptional levels, suggesting that the methylation level quantitatively reflects the regulatory state of the locus (Yu & Lesch, 2024). In the context of *ArsB*, this intermediate methylation likely permits a constitutive baseline expression sufficient to protect the cell during environmental arsenic stress, without the need for dynamic remodeling in response to stress cues (Ribeiro & Lahr, 2022).

Evolutionary Integration of HGT-Derived Genes and Operon-Like Regulation

The epigenetic stability of *ArsB* may also reflect the evolutionary integration status of this horizontally acquired gene within the Galdieria genome. Horizontal gene transfer has been a major driver of adaptation in the Cyanidiophyceae, with up to 5% of the *G. SulPhuraria* genome comprising genes of likely bacterial origin. These acquired genes, particularly those involved in metal and xenobiotic resistance, have substantially contributed to the polyextremophilic phenotype that defines this alga (Schonknecht et al., 2013). However, newly acquired foreign genes face a critical challenge: integration into the host regulatory environment without disrupting cellular function. In bacteria, xenogeneic silencers such as H-NS recognize and selectively suppress the expression of foreign DNA sequences, a mechanism that facilitates the toleration and potential functional integration of acquired genes (Forrest et al., 2022). While eukaryotes employ distinct epigenetic mechanisms distinct from bacterial xenogeneic silencing, the principle remains relevant: foreign genes may require regulatory buffering during integration (Liu et al., 2021).

In the case of *ArsB*, the genomic organization with *ArsB* positioned only 9 bp downstream of the upstream gene (*Gasu_56040*) suggests a prokaryotic-derived operon-like structure (Ribeiro

& Lahr, 2022). The *ArsB* gene likely lacks an independent promoter and may be co-transcribed as part of a larger transcriptional unit controlled by the promoter upstream of Gasu_56040. The upstream gene, putatively encoding a tesmin/TSO1-like protein, may itself require stable expression for proper cellular function. Consequently, the constitutive and stable methylation profile of the *ArsB* operon promoter region may reflect a regulatory strategy designed to maintain consistent expression of the entire operon without perturbation by environmentally driven chromatin remodeling (Schonknecht et al., 2013). By maintaining methylation at an intermediate level that neither fully silences nor maximally activates the locus, the cell ensures that both the upstream regulatory gene and the *ArsB* effector gene maintain functionally adequate expression levels under all environmental conditions (Elliott et al., 2015). This interpretation is consistent with the broader principle that epigenetically silenced or dynamically remodeling genes may impose regulatory conflicts if they are part of multi-gene operons or regulatory networks; hence, constitutively regulated genes benefit from stable epigenetic states that insulate them from environmental noise.

Limitations and Future Directions

This study is limited by examining only two loci with targeted Sanger bisulfite sequencing, which restricts extrapolation to the wider genome. It also lacks expression and protein data for *SulP* and *ArsB*, so functional effects of methylation are inferred rather than directly shown. Future work using genome-wide methylation, transcriptomic, and proteomic approaches, along with long-term field sampling, could clarify how stable or reversible these epigenetic marks are over time and during re-adaptation.

Conclusion

This study demonstrates that DNA methylation serves as a gene-specific, environmentally responsive epigenetic mechanism that fine-tunes the expression of both native and horizontally acquired genes in the polyextremophilic red microalga *G. SulPhuraria*. The hypermethylation of *SulP* in field conditions reflects epigenetic repression of a nutrient transporter in a sulfate-saturated environment, while the constitutive intermediate methylation of *ArsB* ensures continuous protection against unpredictable arsenic stress. These contrasting patterns underscore the nuanced regulatory strategies that eukaryotic extremophiles have evolved to balance competing demands: the need to flexibly regulate nutrient acquisition while maintaining constant vigilance against toxins. These findings extend our understanding of epigenetic adaptation in extreme environments and highlight the importance of methylation-dependent gene regulation in the evolutionary integration of horizontally acquired genes into eukaryotic regulatory networks.

Ethical Statement

This study did not involve any experiments on human participants or the use of human data. All experimental procedures were conducted exclusively on environmental samples and algal cultures, and therefore ethical approval for human or clinical research was not required.

Acknowledgements

The authors gratefully acknowledge the University of Sistan and Baluchestan for providing financial support for this research.

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