

**BABEȘ-BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY**

DOCTORAL THESIS

Summary

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Cluj-Napoca

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Physiological and transcriptional insights into the silver-induced stress response in extremely halophilic members of *Halobacteria* class

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Bioremediation, *Halobacteria*, heavy metal tolerance, oxidative stress, silver nanoparticles, silver resistance, stress response, transcriptomic

Summary:

Chapter I. General introduction

Halophiles are organisms thriving in high salt concentrations, with halophilic archaea of the *Halobacteria* class, known as haloarchaea, dominating environments with salt concentrations above 16%. They display red-orange pigmentation, varied morphology, and diverse metabolic capabilities, including aerobic, heterotrophic, anaerobic, and fermentative respiration. Haloarchaea produce rhodopsins serving various functions, and some engage in sulfur respiration. They possess gas vesicles for oxygen and light acquisition, store nutrients as polyhydroxyalkanoates, and harbor DNA molecules such as megaplasmids and small plasmids encoding essential features (Andrei *et al.*, 2012). The taxonomy of haloarchaea has been recently classified into three orders and six families (Oren, 2019).

Extremophiles, organisms thriving in harsh environments unsuitable for humans, are valuable models for studying adaptation to dynamic conditions, offering insights into biotechnological applications and astrobiological inquiries. These organisms face diverse stressors, including salt concentration fluctuations, pH variations, and high radiation levels. To survive, they employ intricate mechanisms such as osmoregulation strategies like the "salt-in" and "salt-out" mechanisms, membrane adaptations, and stress response gene expression modulation. Halophiles also utilize specific adaptations to counter oxygen deprivation and high irradiance, including gas vesicles for oxygen access and photoprotective mechanisms involving carotenoids and DNA repair systems (Saini *et al.*, 2023).

Stress proteins are crucial for haloarchaea survival in challenging environments. They include proteasomes for targeted protein breakdown, heat shock proteins for preventing misfolding, and universal stress proteins for various cellular functions. Transcription factors regulate gene expression in response to specific stressors. Unique strategies like morphological changes and halomucin secretion aid in stress response. Despite gaps in understanding their functionality, these proteins contribute significantly to haloarchaeal resilience (Matarredona *et al.*, 2020; Llorca and Martínez-Espinosa, 2022).

The response of prokaryotic cells to heavy metals involves a complex interplay of various mechanisms that counteract their toxic effects. Heavy metals have dual functions: while beneficial at lower concentrations for microbial metabolism, they are toxic at higher concentrations, altering cellular structures and processes. Microorganisms utilize diverse strategies for detoxification, including the production of exopolysaccharides, biofilm formation, and sequestration of metals inside and outside the cell (Voica *et al.*, 2016). These mechanisms involve chelating substances, enzymatic reduction, and regulation of gene expression. Prokaryotes employ various transporters to export surplus metals and use molecules like metallothioneins for intracellular sequestration, ensuring metal homeostasis. These adaptive responses enable prokaryotic cells to survive in heavy metal-contaminated environments, emphasizing the importance of understanding microbial capabilities for bioremediation while

highlighting the need for innovative approaches to address heavy metal contamination's ecological and health impacts (Pal *et al.*, 2022).

The antimicrobial properties of silver have re-emerged as a valuable asset in combating antibiotic resistance, offering a promising avenue through silver nanoparticles (AgNPs) production. AgNPs are effective against microorganisms while harmless to hosts, with the ability to gradually release silver ions. Silver disrupts essential cellular processes by targeting cell membranes and thiol groups of cellular proteins. Microorganisms have developed silver tolerance mechanisms, including extracellular sequestration and efflux pumps, while some bacteria employ detoxifying agents like pyocyanin. In biofilms, silver interacts with extracellular substances, altering its toxicity. AgNPs biosynthesis involves cellular compounds or nitrate reductases, with haloarchaea microorganisms favored for synthesis due to their unique cellular structure (Terzioğlu *et al.*, 2022; Mabey *et al.*, 2019).

Aim of the thesis

Silver, widely utilized across various domains, has raised concerns regarding its potential environmental accumulation (Pereira *et al.*, 2013). When present in high concentrations, metals can seriously compromise cellular integrity, prompting the activation of survival mechanisms. Although initial research on silver tolerance in prokaryotes focused on the role of common compounds in safeguarding cellular environments, it failed to account for species-specific tolerance (Voica *et al.*, 2016). Key components of the response to heavy metals encompass ATP-binding cassette transporters and P-type ATPases. Prokaryotes react to metals by increasing the expression of thiol compounds, crucial for maintaining a reducing intracellular milieu. Enzymes such as nitrate reductase might convert toxic silver ions into non-toxic forms (Kaur *et al.*, 2006). A thorough understanding of the interactions between metals and halophilic prokaryotes is imperative for devising strategies to mitigate metal pollution in saline habitats. Overall, unraveling the molecular foundation of metal tolerance in halophilic archaea necessitates interdisciplinary approaches that integrate genomics, molecular biology, and bioinformatics.

The main aim of this doctoral thesis was to explore the molecular and physiological mechanisms underlying heavy metal tolerance in representatives of halophilic archaea from *Halobacteria* class.

To achieve the primary goal of the thesis, we outlined two specific objectives:

1. To physiologically assess silver-resistant halophilic archaeal strains capable of producing silver nanoparticles, while also conducting chemical and physical analyses to characterize the biosynthesized silver nanoparticles. (**Chapter II**).
2. To identify gene expression patterns elicited by exposure to silver ions by employing transcriptomic techniques (**Chapter III**).

Chapter II. Physiological response to silver toxicity in the extremely halophilic archaeon *Halomicrobium mukohataei*

Adaptive strategies responsible for heavy metal tolerance were explored in the extremely halophilic archaeon *Halomicrobium (Hmc.) mukohataei* DSM 12286^T. The tested strain was seemingly able to overcome silver-induced oxidative stress (assessed by malondialdehyde quantification, catalase assay and total antioxidant capacity measurement) mainly through non-enzymatic antioxidants. Energy dispersive spectrometry analysis illustrated the presence of colloidal silver in *Hmc. mukohataei* cultures exposed to AgNO₃. Bright-field and transmission electron microscopy images, as well as dynamic light scattering analysis, demonstrated the presence of intracellular nanoparticles, mostly spherical, within a size range of 20-100 nm. As determined by the zeta potential measurement, the biosynthesized nanoparticles were highly stable, with a negative surface charge.

Our research is a first attempt in the systematic study of the oxidative stress and intracellular silver nanoparticle accumulation, generated by exposure to silver ions, in members of *Halobacteria* class, thus broadening our knowledge on mechanisms supporting heavy metal tolerance of microbial cells living under saline condition.

Chapter III. Genome-wide transcriptional response to silver stress in extremely halophilic archaeon *Haloferax alexandrinus* DSM 27206^T

The extremely halophilic archaeon *Haloferax (Hfx.) alexandrinus* DSM 27206^T was previously documented for the ability to biosynthesize silver nanoparticles while mechanisms underlying its silver tolerance were overlooked. In the current study, we aimed to assess the transcriptional response of this haloarchaeon to varying concentrations of silver, seeking a comprehensive understanding of the molecular determinants underpinning its heavy metal tolerance

The growth curves confirmed the capacity of *Hfx. alexandrinus* to surmount silver stress, while the SEM-EDS analysis illustrated the presence of silver nanoparticles in cultures exposed to 0.5 mM silver nitrate. The RNA-Seq based transcriptomic analysis of *Hfx. alexandrinus* cells exposed to 0.1, 0.25, and 0.5 mM silver nitrate revealed the differential expression of multiple sets of genes potentially employed in heavy-metal stress response, genes mostly related to metal transporters, basic metabolism, oxidative stress response and cellular motility. The RT-qPCR analysis of selected transcripts was conducted to verify and validate the generated RNA-Seq data. Our results indicated that *copA*, encoding the copper ATPase, is essential for the survival of *Hfx. alexandrinus* cells in silver-containing saline media. The silver-exposed cultures underwent several metabolic adjustments that enabled the activation of enzymes involved in the oxidative stress response and impairment of the cellular movement capacity. To our knowledge, this study represents the first comprehensive analysis of gene expression in halophilic archaea facing increased levels of heavy metals.

Chapter IV. General conclusions and originality of the results

IV.1. General conclusions

The pollution of saline environments by metallic ions due to urbanization and industrial expansion is a significant ecological and health issue. To address this, halophilic archaea, which can produce silver nanoparticles, were studied for their adaptive mechanisms. This doctoral thesis aimed to assess the physiological reaction of the silver-resistant strain *Halomicrobium mukohataei* DSM 12286^T and identify gene expression patterns triggered by exposure to silver ions in the *Haloferax alexandrinus* DSM 27206^T strain.

The study investigated the ability of the haloarchaeon *Halomicrobium mukohataei* DSM 12286^T to handle increasing Ag⁺ concentrations. Non-enzyme-based antioxidants were found to reduce silver oxidative stress. *Hmc. mukohataei* cells exposed to AgNO₃ produced colloidal silver nanoparticles with a spherical shape and size ranging from 20 to 100 nm. The zeta potential analysis revealed strong stability and a negatively charged surface charge in the nanoparticles (**Chapter II**).

Moreover, we analyzed the transcriptomes of *Haloferax alexandrinus* DSM 27206^T cells exposed to different concentrations of silver, revealing distinct patterns of gene expression related to heavy metal stress. The response involved changes in gene expression related to transporting metals, fundamental metabolic processes, combating oxidative stress, and facilitating cellular movement. The CopA copper ATPase was identified as a key player in regulating metal ion movement within cells. The cells adapted to challenging conditions by adjusting their metabolism, reallocating resources efficiently, and activating stress response mechanisms.

Overall, the research investigated the adaptive mechanisms of halophilic archaea strains, specifically those tolerant of elevated silver ion concentrations, to cope with heavy metals. Through physiological and genetic examinations, the study reveals the physiological, biochemical, and genetic mechanisms underlying the silver stress response in haloarchaea, namely *Halomicrobium mukohataei* DSM 12286^T and *Haloferax alexandrinus* DSM 27206^T. This knowledge provides insights into their tolerance mechanisms and lays the groundwork for future bioremediation efforts in silver-contaminated environments.

IV.2. Originality and novelty of the results

The thesis introduces original findings in *Halobacteria* research. Firstly, unprecedented in the existing literature, it explores how *Halomicrobium mukohataei* DSM 12286^T, a *Halobacteria* class member, combats silver-induced oxidative stress by increasing non-enzymatic antioxidants, thus shedding light on stress response mechanisms in these microorganisms. Secondly, it documents the first

instance of silver nanoparticles biosynthesis within *Halomicrobium mukohataei*, expanding our understanding of *Halobacteria*'s response to silver stress (**Chapter II**).

Moreover, this study presents the first in-depth examination of the transcriptional profile of haloarchaea in response to high metal concentrations. Specifically, our unparalleled results revealed that the survival of *Haloferax alexandrinus* in silver-rich saline environments consistently hinges on the presence of the copper ATPase encoded by *copA*. Through RNA-seq analysis, we further uncovered for the first time in the known literature, the activation of antioxidant enzymes and the downregulation of motility-related genes in *Haloferax alexandrinus* when exposed to heightened levels of silver ions. These findings significantly enhance our understanding of the adaptive mechanisms employed by halophilic archaea to withstand elevated heavy metal stress (**Chapter III**).

IV.3. Perspectives

Future investigations in this research field could delve into the genetic control underlying the synthesis of silver nanoparticles in *Halomicrobium mukohataei* and *Haloferax alexandrinus*, unveiling molecular mechanisms and potential utilities in bioremediation and biomedical contexts. Comparative genomic analyses among haloarchaeal species might uncover shared and distinctive adaptive tactics to heavy metal stress. This endeavor might imply assessing the presence, diversity, distribution and roles of genes implicated in metal transport, detoxification pathways, and stress response mechanisms across diverse haloarchaeal species. Moreover, broadening transcriptomic assessments to encompass a wider array of environmental conditions in combination with elevated heavy metal levels could enhance our knowledge of haloarchaea's adaptive responses to various environmental and man-generated stressors. It therefore may be of stringent interest to scrutinize gene expression patterns under varying concentrations and types of heavy metals, along with exploring the interplay between heavy metal stress and other environmental elements. Additionally, probing into the potential for genetic alteration or engineering of haloarchaeal strains to improve their tolerance and efficacy in heavy metal bioremediation processes could present a promising avenue for forthcoming inquiries. This could encompass exploring genetic editing methodologies to modify crucial genes involved in heavy metal tolerance pathways and evaluating the repercussions of these alterations on the strains' viability and growing in polluted environments. Lastly, examining the potential applications of silver nanoparticles in bioremediation or biomedical domains would offer valuable tools.

Publications during PhD stage

List of publications included in the thesis as chapters

Chapter II

Buda, D.M., Bulzu, P.A., Barbu-Tudoran, L., Porfire, A., Pătraș, L., Sesărman, A., Tripon, S., Șenilă, M., Ionescu, M.I., Banciu, H.L., 2019. Physiological response to silver toxicity in the extremely halophilic archaeon *Halomicrobium mukohataei*. *FEMS Microbiol Lett*, 366(18), p.fnz231. <https://doi.org/10.1093/femsle/fnz231>

Chapter III

Buda, D.M., Szekeres, E., Tudoran, L.B., Esclapez, J., Banciu, H.L., 2023. Genome-wide transcriptional response to silver stress in extremely halophilic archaeon *Haloferax alexandrinus* DSM 27206^T. *BMC Microbiol*, 23(1), p.381. <https://doi.org/10.1186/s12866-023-03133-z>

List of publications not included in the thesis

1. **Buda, D.M.**, Bulzu P.-A., Cristea, A., Banciu, H.L. (2019) The saline aquatic systems as ‘natural reservoirs’ for microorganisms with current and potential applications. *Aerul Apa Compon Mediu*, ISSN 2067-743X. pp. 427-434. http://doi.org/10.24193/AWC2019_42.
2. Bulzu, P.-A., Cristea, A., **Buda, D.M.**, Banciu, H.L. (2019) Asgardarchaeota – a novel prokaryotic group discovered in aquatic sediments that might shed light on the origin and early evolution of Eukaryotes. *Aerul Apa Compon Mediu*, ISSN 2067-743X. pp. 435-446. http://doi.org/10.24193/AWC2019_43.
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