Response of moderately halophilic bacterium *Salinicola socius* strain SMB35^T cells to oxidative stress induced by the action of hydrogen peroxide

Lyudmila Anan'ina[1] [1] and *Alexey* Gorbunov[2]

[1]Institute of Ecology and Genetics of Microorganisms UB RAS – branch of the Perm Federal Research Center of the UB RAS, 614081 Perm, Russia
[2]Institute of Technical Chemistry UB RAS – branch of the Perm Federal Research Center of the UB RAS, 614013 Perm, Russia

Abstract: The aim of the present study was to investigate the response of *Salinicola socius* strain SMB35^T cells to oxidative stress modelled by the introduction of hydrogen peroxide into the medium. It was found that when hydrogen peroxide was added to the cell suspension to a final concentration of 25 μM, a slight decrease in biomass was observed, while the addition of hydrogen peroxide to a concentration of 25 mM resulted in a significant decrease in biomass. Since a statistically significant decrease in biomass was noted upon exposure to hydrogen peroxide at a concentration of 25 mM, the amount of compatible solutes in cells of *Salinicola socius* strain SMB35^T after exposure to hydrogen peroxide was evaluated. Cell extracts were studied by proton magnetic resonance spectroscopy. Under conditions of oxidative stress, a trend towards a decrease in the amount of ectoine was observed, and the pool of its precursor – N(4)-acetyl-L-2,4-diaminobutyrate - decreased by a factor of 2.1. Oxidative stress significantly affected the intracellular amount of glutamate; its content decreased by 5.6 times. It was found that after incubation with H_2O_2, the amount of alanine and valine in the cells reduced. In response to oxidative stress, a trend towards increasing trehalose content in cells was observed, but its amount was 8.7 times less than that of ectoine.

1 Introduction

Reactive Oxygen Species (ROS) are highly reactive compounds formed through the partial reduction of molecular oxygen (O_2) during biochemical and photochemical processes. Their generation is linked to oxygen's ability to accept electrons, leading to the production of both radical (containing unpaired electrons) and non-radical forms [1].

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1 Corresponding author: ludaananyina@mail.ru

In natural ecosystems, ROS originate from both abiotic and biotic sources. In soils, ROS can form via the photosensitization of soil organic components and possibly minerals (e.g., titanium and zinc oxides) or resulted indirectly from the reaction of water with humic substances in the excited triplet state or from degradation of H_2O_2 by transition metal cations such as Fe^{3+} via a photo-Fenton reaction —is hypothesized [2]. Interestingly, ROS formation is observed even in aphotic (light-deprived) soil layers, suggesting alternative synthesis pathways such as microbial activity or redox cycles involving iron and organic matter [1].

In living organisms, ROS are natural byproducts of aerobic metabolism. Under normal conditions, their concentration is tightly regulated by antioxidant systems, including enzymes (superoxide dismutase, catalase, peroxidases) and low-molecular-weight antioxidants (glutathione, carbohydrates). However, abiotic stresses (UV radiation, salinity, heavy metals) disrupt this balance, leading to excessive ROS generation and oxidative damage to lipids, proteins, and DNA [3]. Among these stresses, salinity is a critical challenge.

Soil salinization, a major driver of land degradation, reduces soil fertility and contributes to desertification in global drylands. Irrigation-induced salinization affects approximately 60 million hectares, or 24% of the world's irrigated land [4]. Meanwhile, the role of bacteria in mitigating erosion is significant. Halophilic bacteria contribute to organic matter decomposition, promoting humus formation, and produce substances (e.g., polysaccharides) that bind soil particles into aggregates resistant to wind and water erosion. Thus, understanding the oxidative stress defence mechanisms of bacteria inhabiting saline ecosystems is crucial. Their adaptation supports microbial community resilience and prevents ecosystem collapse.

Bacteria of the genus S*alinicola* (family *Halomonadaceae*) are halophiles inhabiting diverse saline ecosystems, including soils. They also colonize the rhizosphere of halophytes (salt-adapted plants), forming symbiotic associations. These interactions involve: influence plant growth and development by degrading toxic organic compounds, increasing the availability of mineral elements, modulating the production or degradation of phytohormones, inhibiting pathogens or providing plants with organic compounds that promote salt tolerance [5].

A key adaptation of *Salinicola* is the accumulation of compatible solutes—ectoine and hydroxyectoine—which stabilize cellular structures under osmotic stress. However, their role in ROS neutralization remains poorly understood. Unlike model non-halophilic bacteria (e.g., *Acinetobacter baumannii* and *Xanthomonas axonopodis* pv. *citri*), halophiles may combine traditional enzymatic antioxidant pathways with unique osmolytic strategies.

Despite the ecological importance bacteria of *Salinicola* genus, their adaptation to oxidative stress remains unexplored. This study is the first to investigate changes in the metabolomic profile of *Salinicola socius* SMB35^T under hydrogen peroxide exposure—a key non-radical ROS. Analyses included: (1) quantitative assessment of osmolyte pools (ectoine, trehalose); and (2) genomic screening for antioxidant enzyme genes (superoxide dismutase, catalase).

2 Materials and methods

2.1 Object of the study

The strain of *S. socius* SMB35^T was used in this work. The strain was previously isolated from salt-affected soil.

2.2 Preparation of biomass for extraction of organic compounds

The strain under investigation was cultivated in Raymond's mineral medium (RMM) containing (g/L) NH_4NO_3 (2.0), $MgSO_4 * 7H_2O$ (0.2), KH_2PO_4 (2.0), Na_2HPO_4 (3.0), $CaCl_2 * 6H_2O$ (0.01), Na_2CO_3 (0.1), 2 ml of 1% $MnSO_4 * 5H_2O$ and 1 ml of 1% $FeSO_4 * 7H_2O$. The RMM was supplemented with 2% NaCl. The substrate was glucose at a final concentration of 1 g/L. The bacterial culture was grown in 100 ml of medium in 250 ml Erlenmeyer flasks. Incubation was carried out under stationary conditions without stirring the culture medium. The incubation temperature was 25°C. The inoculum (1% vol/vol) was the culture grown to stationary growth phase under the conditions described above.

Optical density was measured on a BioSpec-mini UV/VIS spectrophotometer (Shimadzu Corporation, Japan) at 540 nm in a cuvette with an optical path length of 1 cm.

At the end of the incubation period, the cell suspension was divided into equal parts by volume. Hydrogen peroxide was added to one of them to a final concentrations of 25 μM or 25 mM. After 2 hours the cells were precipitated by centrifugation on a 3K30 centrifuge (Sigma, Germany) at 10000 g for 10 minutes at the same temperature as in the experiment. Extraction of organic compounds from the cells was performed with 80% ethanol solution. The dried extracts were stored at 5ºC until analysis. The cell sediment obtained after the extraction was dried and the mass was determined.

2.3 Proton Magnetic Resonance Spectroscopy

Spectra were recorded on a Bruker Avance Neo 400 instrument (Bruker Corporation, USA) equipped with a 5 mm broadband BBO probe with a Z-gradient and a sample temperature control system with a temperature setting accuracy of 0.1°C. *Tert*-butyl alcohol was added to the sample as an internal standard in the amount of 0.09-0.10 mg/sample. Chemical shifts (δ) are given in ppm and are measured relative to the signal of the methyl groups of *tert*-butyl alcohol (1.24 ppm.).

Preliminary identification of signals in the spectrum was performed by comparison with spectra of compounds from the Biological Magnetic Resonance Data Bank database [6]. The identification of compounds was confirmed by sequential addition of the reference compounds ectoine (Fluka, Germany), glutamate, alanine, valine and trehalose (Sigma, Germany) to the solutions under investigation and subsequent recording of the spectrum. The only exception was N(4)-acetyl-L-2,4-diaminobutyrate (NADA), whose signals were identified by comparison with previously published data [7].

The chemical shifts of the peaks used for the integration were 2.23 ppm for ectoine (CH_3), 2.35 ppm for glutamate (β-CH_2), 1.47 ppm for alanine (CH_3), 1.03 ppm for valine (CH_3, the left doublet), 1.99 ppm for NADA (CH_3) and 5.19 ppm for trehalose (anomeric proton). The concentration of a compound in a sample was determined by comparing the area of its non-overlapping peak with the peak area of the internal standard [8]. The amounts of compounds were recalculated per mg of dry weight of biomass remaining after extraction (mg DW).

2.4 Search for genes in the genome of *S. socius* SMB35^T

The search for genes encoding catalases and superoxide dismutases was carried out in the genome of *S. socius* SMB35^T, deposited in Genbank under the number BioProject PRJNA357614 [9].

2.5 Statistical processing

For statistical processing of the data, the arithmetic mean and standard deviation of two independent experiments were determined using the Microsoft Office Excel 2003 module.

3 Results

3.1 Effect of different concentrations of hydrogen peroxide on *S. socius* SMB35^T cells

The effect of different concentrations of hydrogen peroxide on *S. socius* SMB35^T cells was studied. Hydrogen peroxide was added to the cell suspension to a final concentration of 25 µM and 25 mM. It was shown that when hydrogen peroxide was added to the cell suspension to a final concentration of 25 µM, a slight decrease in biomass was observed. It amounted to 98.5% of the biomass not exposed to ROS (Fig.1). Application of hydrogen peroxide one thousand times higher than the concentration of 25 µM resulted in biomass reduction by 31.3% compared to the variant without ROS exposure.

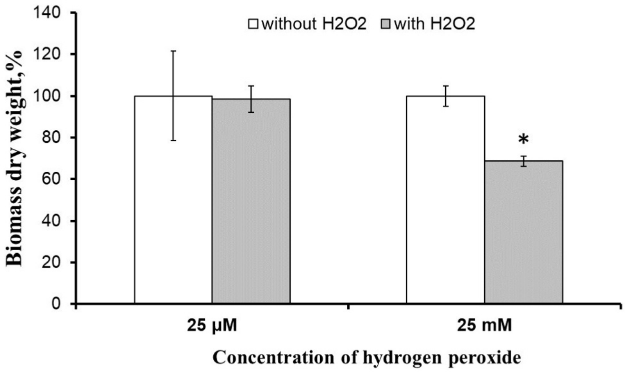

Fig1. Dry weight of biomass after exposure of cell suspension to different concentrations of hydrogen peroxide. * – difference between the experiment and the control is significant at $p \leq 0.01$.

3.2 Compatible solutes accumulated by *S. socius* SMB35^T cells

Proton magnetic resonance spectroscopy of extracts from *S. socius* strain SMB35^T cells not exposed to hydrogen peroxide revealed the presence of signals assigned to ectoine, NADA, trehalose, glutamate, alanine, and valine (Table). Ectoine and glutamate were present in the highest amounts in the cells of the studied bacterium, but ectoine was more concentrated than glutamate. The amounts of ectoine and glutamate were 91.0±2.1 and 65.0±3.3 nmol/mg DW, respectively. Notably, *S. socius* strain SMB35^T cells synthesized ectoine, a widespread osmoprotectant in the prokaryotic world, when sodium chloride was added to the medium at a rather low concentration of only 2%. In smaller quantities, *S. socius* strain SMB35^T cells accumulated such compatible solutes as NADA (8.3±0.6 nmol/mg DW) and trehalose (4.1±3.1 nmol/mg DW).

Table. Intracellular levels of metabolites before exposure of *S. socius* SMB35^T cells to hydrogen peroxide.

Compound	glutamate	alanine	valine	ectoine	NADA	trehalose
Intracellular content (nmol/mg DW)	65.0± 3.3	13.9± 0.1	11.2±0.7	91.0±2.1	8.3±0.6	4.1±3.1

Means and standard deviations are given.

3.3 Effect of oxidative stress on the amounts of compatible compounds in *S. socius* SMB35^T cells

Since a statistically significant decrease in biomass was noted upon exposure to hydrogen peroxide at a concentration of 25 mM, the amount of compatible solutes in cells of *S. socius* strain SMB35^T after exposure to hydrogen peroxide was evaluated.

An interesting fact was revealed - a trend towards a decrease in the intracellular amount of ectoine after exposure to hydrogen peroxide was observed (Fig. 2). The decrease in its amount was 10.7%. However, the addition of hydrogen peroxide had a greater effect on the content of ectoine's precursor - NADA. Its content decreased by 2.1-fold. Trehalose amount after the addition of hydrogen peroxide increased in all repeats of the experiment, but a high variation in its content was noted.

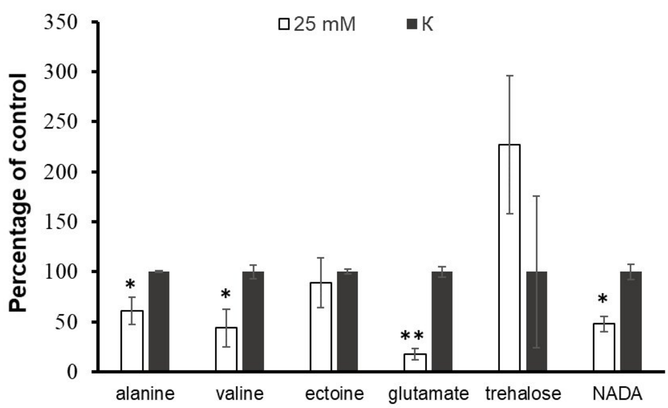

Fig 2. Effect of hydrogen peroxide in 25 mM concentration on the amounts of compatible compounds in *S. socius* SMB35^T cells. * – difference between the experiment and the control is significant at $p \leq 0.05$, ** – difference between the experiment and the control is significant at $p \leq 0.01$.

After hydrogen peroxide treatment, the levels of some biomolecules involved in central metabolism were altered. Oxidative stress had a major effect on the amount of glutamate, whose pool decreased 5.6-fold (Fig. 2). The enzyme systems for alanine and valine synthesis responded differently to the effects of hydrogen peroxide on the cells. The amount of alanine decreased 1.6-fold, while the amount of valine decreased to a much greater extent by 2.3-fold (Fig.2).

3.4 Search for genes encoding catalase and superoxide dismutase in the *S. socius* SMB35^T genome

A gene encoding copper- and zinc-containing superoxide dismutase (Cu/Zn SOD, SODC) (OLO04089) is present in the genome. In addition, the *S. socius* SMB35^T chromosome contains a gene encoding manganese superoxide dismutase (SODA) (OLO02972). In addition to the above, the genome contains three genes encoding catalases. In the *S. socius* SMB35^T genome, genes encoding monofunctional heme-containing catalase (KatEs) (OLO04654) and putative manganese-containing catalase (MnCATs) (OLO05202.1) were identified. A gene for a catalase-like heme-binding protein similar to the unstudied srpA protein is present in the genome also (OLO04053.1).

4 Discussion

In experiments to study the effect of ROS on bacterial cells, hydrogen peroxide is usually used at a concentration of 25-50 μM. At the same time, for the phytopathogenic bacterium *Xanthomonas axonopodis* pv. *citri*, which causes citrus cancer, it was shown that its cells can withstand hydrogen peroxide at concentrations up to 30 mM [10]. Considering the above facts, in the present work, after the cell suspension of *S. socius* strain SMB35^T reached an optical density value of 0.3 OD, hydrogen peroxide was added to it to a final concentration of 25 μM and 25 mM. It was found that when hydrogen peroxide was added to the cell suspension to a final concentration of 25 μM, a slight decrease in biomass was observed, while the addition of hydrogen peroxide to a concentration of 25 mM resulted in a significant decrease in biomass (Fig.1). The results obtained may indicate cell death and destruction of *S. socius* strain SMB35^T cells when exposed to hydrogen peroxide at 25 mM concentration (Fig.1). Similar results were obtained earlier for *X. axonopodis* pv. *citri*. At the action of hydrogen peroxide at a concentration of 30 mM, the survival rate of bacterial cells was 40% [10]. It is worth emphasizing that a similar decrease in the amount of biomass of the moderately halophilic culture *S. socius* strain SMB35^T under the action of hydrogen peroxide at a concentration of 25 mM was noted in the exponential growth phase. Probably, such an effect can be explained by the presence of compatible compounds in the cells of the halophilic culture, including ectoine and NADA. These compounds protect cell macromolecules from adverse effects by maintaining their hydration shell.

It should be noted that of all the *S. socius* strain SMB35^T cell compatible compounds identified by NMR, only trehalose showed a trend for an increase in the intracellular amount in response to oxidative stress induced by hydrogen peroxide. Some carbohydrates, including trehalose, can perform antioxidant functions within cells [11]. During oxidative stress, trehalose acts primarily as a free radical scavenger, thus blocking their interaction with proteins and lipids [11].

Under conditions of oxidative stress, the amount of glutamate decreased in the cells. This may be because glutamate is used to synthesize glutathione, a key molecule in maintaining intracellular redox potential and protecting the bacterium from reactive oxygen species such as hydrogen peroxide [12]. It is likely that the decrease in glutamate led to a decrease in NADA and ectoine, since glutamate is involved in the synthesis of these compounds. The literature presents data on the effect of oxidative stress induced by exposure of gram-negative prokaryote *Acinetobacter baumannii* cells to laser. At the lethal dose, a decrease in the amount of alanine was observed in the cells, the content of valine did not change. However, in *A. baumannii* cells, oxidative stress led to an increase in glutamate [13]. The decrease in alanine and valine may be related to the need to replenish the pyruvate pool. A decrease in the amount of pyruvate has been observed in the cells of some bacteria under conditions of oxidative stress [13]. The researchers linked this effect to the fact that pyruvate is a powerful and efficient ROS scavenger [13].

In the genome of *S. socius* SMB35^T, a search for genes encoding enzymes involved in the detoxification of reactive oxygen species was carried out. Genes encoding such enzymes as superoxide dismutases were identified. The enzymes can convert superoxide anion free radicals into hydrogen peroxide and molecular oxygen, protecting organism from hydroxyl free radicals. Notably, the chromosome carries superoxide dismutase genes from different families. A gene encoding copper- and zinc-containing superoxide dismutase (SODC) (OLO04089) is present in the genome. In addition, the *S. socius* SMB35^T chromosome contains a gene encoding the manganese superoxide dismutase (SODA) (OLO02972). In *Escherichia coli,* superoxide dismutase A is an inducible enzyme related to oxidative stress such as H_2O_2 [14]. In addition to the above, the genome contains three genes encoding catalases. This enzyme is the main sink of H_2O_2, catalyzing its cleavage to water and oxygen. Based on phylogenetic analysis, three distinct families of catalases can be distinguished: typical (monofunctional) heme-containing catalases (KatEs), bifunctional heme-containing catalase-peroxidase (KatGs), and (non-heme) manganese-containing catalases (MnCats) [10]. Genes encoding monofunctional heme-containing catalase (KatEs) (OLO04654) and putative manganese-containing catalase (MnCATs) (OLO05202.1) have been identified in the *S. socius* SMB35^T genome. A gene for a catalase-like heme-binding protein similar to the unstudied srpA protein in the genome also presents (OLO04053.1). Earlier, using *X. axonopodis* pv. *citri* as an example it was demonstrated that the expression of genes encoding catalases changes in response to any factors related to microenvironment [10].

5 Conclusions

Saline ecosystems are a typical habitat for bacteria of the *Salinicola* genus. In addition to osmotic imbalance, salinization of the environment causes oxidative damage to cell components, since an increase in the concentration of ions (e.g. Na^+, Cl^-) disrupts the homeostasis of cellular ions, stimulating the generation of ROS.

Analysis of the genome of the bacterium *S. socius* SMB35^T revealed the presence of genes encoding such enzymes as catalases and superoxide dismutases involved in the neutralization of ROS at different stages of their formation. Moreover, genes encoding isoenzymes of both catalases and superoxide dismutases were found in the genome. Notably, the *S. socius* SMB35^T genome contains a gene encoding a copper- and zinc-containing superoxide dismutase. Due to their location in the periplasm or membrane-bound form, these enzymes protect bacteria from oxygen radical damage and promote their survival when exposed to exogenous reactive oxygen species [15]. Thus, the cells of the *S. socius* SMB35^T strain possess enzyme systems for the neutralization of ROS formed both inside the cell and acting from outside.

Therefore, in the present work, the pool of osmoprotective compounds of the halophilic bacterium *S. socius* strain SMB35^T was studied under oxidative stress conditions. It was shown that oxidative stress induced metabolic reorganization in cells of the *S. socius* strain SMB35^T. On the one hand, the carbon flow was directed to replenish the pools of glutamate and pyruvate, while the catabolism of amino acids and their derivatives was triggered. A decrease in the amount of ectoine and its precursor, NADA, may indicate that their oxidation was used to replenish the pools of glutamate. On the other hand, the process of trehalose synthesis was stimulated, leading to an increase in its level.

The present study showed that *S. socius* strain SMB35^T cells may use two main strategies for protection against ROS: (1) antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT) and (2) small antioxidant molecules (trehalose) that neutralize or prevent ROS.

Thus, *S. socius* strain SMB35^T cells have different defence mechanisms against oxidative stress.

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