MICROBIOLOGY AND VIROLOGY

EXPRESSION OF THE *soxRS* REGULON IN BACTERIAL CELLS EXPOSED TO VARIOUS STRESS FACTORS

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ABSTRACT

Background. *Some stress responses contribute to the formation of bacterial antibiotic resistance, including the soxRS oxidative defense regulon. Elevation of reactive oxygen species production and oxidative stress was detected in bacterial cells exposed to various environmental stresses. It can be supposed that a stress-mediated increase in the level of reactive oxygen species will activate the expression of the soxRS regulon genes, which may provide pre-adaptation to antibiotics.*
The aim. *To study changes in the expression of soxRS regulon genes in Escherichia coli cells exposed to NaCl, acetic acid, and heating.*
Materials and methods. *Gene expression was measured in cells bearing reporter gene fusions (soxS::lacZ, nfo::lacZ). An overnight broth culture was diluted in fresh LB broth to OD600 = 0.1 and cultivated at 37 °C without stirring until OD600 = 0.3, then the stressors were applied.*
Results. *Exposure to NaCl and acetic acid activated the expression of soxRS regulon genes, while heating caused a decrease in gene expression. An increase in the expression level was observed in cells subjected to stresses of low intensity (which did not cause a decrease in the number of colony-forming units (CFU) by the 4th hour of exposure compared to the beginning of the stress exposure) and medium intensity (which caused a 10-fold decrease in the number of CFU), whereas high-intensity stresses (which caused a decrease in the number of CFU by more than 10 times), regardless of their nature, were accompanied by a decrease in the expression of the soxRS regulon genes.*
Conclusion. *Under the conditions studied, only the osmotic stress caused by the addition of NaCl was accompanied by a significant activation of the soxRS regulon genes. Sublethal exposure to NaCl, causing an increase in the expression of soxRS regulon genes by 2–2.5 times, may provide pre-adaptation of bacteria to the factors that this regulon is aimed at counteracting, including antibacterial drugs.*

Key words: *osmotic shock, acid stress, heat shock, oxidative stress, antibiotics, soxS*

Received: 17.10.2022
Accepted: 17.02.2023
Published: 05.05.2023

For citation: Akhova A.V., Tkachenko A.G. Expression of the *soxRS* regulon in bacterial cells exposed to various stress factors. *Acta biomedica scientifica*. 2023; 8(2): 117-123. doi: 10.29413/ABS.2023-8.2.11

ЭКСПРЕССИЯ ГЕНОВ *soxRS*-РЕГУЛОНА В КЛЕТКАХ БАКТЕРИЙ, ПОДВЕРГНУТЫХ ДЕЙСТВИЮ РАЗЛИЧНЫХ СТРЕСС-ФАКТОРОВ

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РЕЗЮМЕ

Актуальность. *В формирование устойчивости бактерий к антибиотикам вносят вклад различные адаптивные механизмы, в том числе гены защитного ответа на окислительный стресс, объединённые в soxRS-регулон. В стрессовых условиях в клетках бактерий происходит повышение продукции активных форм кислорода и развитие окислительного стресса. Можно предположить, что повышенный уровень активных форм кислорода будет активировать экспрессию генов soxRS-регулона, что может обеспечить преадаптацию бактерий к воздействию антибиотиков.*

Цель. *Исследовать изменение экспрессии генов, входящих в soxRS-регулон, в клетках Escherichia coli, подвергнутых действию NaCl, повышенных температур и уксусной кислоты.*

Материалы и методы. *Уровень экспрессии генов определяли с использованием штаммов E. coli, несущих репортерные генные слияния промотора исследуемого гена (soxS, nfo) со структурной частью гена lacZ, в условиях периодического культивирования в бульоне LB без перемешивания.*

Результаты. *Активацию экспрессии генов soxRS-регулона вызывало воздействие NaCl и уксусной кислоты, а тепловой шок сопровождался снижением генной экспрессии. Увеличение уровня экспрессии наблюдалось в клетках, подвергнутых стрессам низкой (не вызывавшим снижения количества колониеобразующих единиц в культуре к четвёртому часу воздействия по сравнению с началом стрессового воздействия) и средней интенсивности (вызывавшим снижение количества колониеобразующих единиц на порядок), а стрессовые воздействия высокой интенсивности (вызывавшие снижение количества колониеобразующих единиц более чем на порядок) вне зависимости от их физико-химической природы сопровождались снижением экспрессии генов soxRS-регулона.*

Заключение. *В исследованных условиях только осмотический стресс, вызванный внесением NaCl, сопровождался значимой активацией генов, входящих в soxRS-регулон. Сублетальное воздействие NaCl, вызывая повышение экспрессии генов soxRS-регулона в 2–2,5 раза, может обеспечивать преадаптацию бактерий к факторам, на противодействие которым направлен данный регулон, в том числе к антибактериальным препаратам.*

Ключевые слова: *осмотический шок, кислотный стресс, нагревание, окислительный стресс, антибиотики, soxS*

Статья поступила: 17.10.2022
Статья принята: 17.02.2023
Статья опубликована: 05.05.2023

Для цитирования: Ахова А.В., Ткаченко А.Г. Экспрессия генов *soxRS*-регулона в клетках бактерий, подвергнутых действию различных стресс-факторов. *Acta biomedica scientifica*. 2023; 8(2): 117-123. doi: 10.29413/ABS.2023-8.2.11

The formation of resistant forms of microorganisms is the reason for the reduced effectiveness of antibiotic therapy. The mechanisms underpinning drug resistance include target alteration or protection, modification and inactivation of the antimicrobial compound, rearrangement of metabolic pathways, or restriction of antibiotic accumulation in the microbial cell (by reducing the transport of the drug into the cell and increasing its active release from the cell) [1–3].

Various mechanisms of defense responses to natural stress factors may be involved in the adaptation of bacteria to antibiotic drugs [4, 5]. In particular, in response to antibiotic exposure, the expression of *soxRS* regulon genes that protect bacteria from oxidative stress is activated. The increased baseline level of expression of this regulon in some cases results in clinically relevant antibiotic resistance in bacteria [6–9].

SoxRS regulon is a two-stage control system. The SoxR protein enters the active form and triggers the expression of the *soxS* gene; the newly synthesised SoxS protein then activates the expression of other genes within this regulon. The SoxR protein is activated by one-electron oxidation of its [2Fe-2S] clusters or their nitrosylation by reactive nitrogen species [10–13]. The *soxRS* regulon includes genes encoding superoxide dismutase that neutralizes superoxide anions (*sodA*), endonuclease involved in DNA repair (*nfo*), isoforms of enzymes resistant to oxidative damage (*fumC*, *acnA*), iron transport regulator (*fur*), proteins limiting the accumulation of hydrophilic xenobiotics in the cell (*tolC*, *micF*, *acrAB*), proteins presumably involved in the maintenance of the reduced form of iron-sulfur sites of enzymes (*fldAB*, *fpr*), and other proteins with unknown functions [14].

It is known that exposure to antibacterial drugs and natural stress factors of different nature causes increased production of free radicals and development of oxidative stress in bacterial cells. While the role of reactive oxygen species and their contribution to the death of cells exposed to various stress factors remains a debatable issue, the accumulation of free radicals caused by stress factors not directly related to their production has been confirmed by numerous publications [15–20]. Many of these stressors, e. g. high osmolarity of the medium, heating, exposure to ethanol and short-chain fatty acids, are used as antimicrobial treatments or preservatives. If these stressors cause induction of *soxRS* regulon, their sublethal effects may contribute to the pre-adaptation of bacteria to antibiotic exposure.

In this study, the expression of the *soxRS* regulon genes was studied in *Escherichia coli* cells exposed to sodium chloride, elevated temperatures and acetic acid (CH3COOH) using the gene fusion method.

MATERIALS AND METHODS

Objects of the study and cultivation conditions. *Escherichia coli* strains carrying transcriptional gene fusions were used as study objects. *E. coli* EH40 strain (GC4468, but *soxS::lacZ*) was kindly provided by B. Demple [21], *E. coli* N9213 strain (GC4468, but *nfo::lacZ* Δmar *rob::kan*) was kindly provided by R.G. Martin [22].

Bacteria maintained on LB slant agar were transferred to 5 ml of LB broth and cultured without agitation at 37 °C for 5–6 h. The grown cells were transferred into 50 ml of LB broth and cultured at 37 °C for 14–16 h. The bacterial culture was then diluted in fresh nutrient medium to an optical density measured at a wavelength of 600 nm (OD600) of 0.1 and cultured under the conditions described above. Once the bacterial culture reached OD600 = 0.3, it was exposed to stressors. Sodium chloride and acetic acid were added to the bacterial culture and the culture was placed on a water bath with appropriate temperature to reproduce heat shock.

The gene expression level was determined using reporter gene fusions of the promoter of the studied gene and the structural part of the *lacZ* gene encoding β-galactosidase. It is assumed that the amount (activity) of the reporter protein is directly proportional to the expression level of the studied gene. β-galactosidase activity was measured in cells pretreated with a mixture of sodium dodecyl sulfate and chloroform using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. β-galactosidase activity was determined and calculated (in Miller units) according to the standard protocol proposed by J. Miller [23].

Bacterial culture density was estimated by measuring its OD600 using a UV1280 spectrophotometer (Shimadzu, Japan) and a cuvette with 10 mm optical path.

The number of colony-forming units (CFUs) was determined by plating on the surface of LB agar in Petri dishes. The number of colonies formed was counted after incubation at 37 °C for 16–18 h.

Statistical data processing was performed using Statistica 6.0 software package (StatSoft Inc., USA). Data are presented as mean and standard error of the mean calculated from at least three independent experiments. The statistical significance of the differences between the mean values of the compared groups was determined using unpaired t-test at $p \leq 0.050$.

RESULTS AND DISCUSSION

Osmotic stress was caused by addition of sodium chloride, acid shock was induced by addition of acetic acid, and heat stress was induced by heating from 37 to 42–55 °C. The effect of these stresses of different intensities on the expression of the *soxS* gene, which encodes a transcriptional regulator responsible for the activation of genes of the regulon, and its target gene *nfo*, which encodes a DNA repair enzyme, was studied. The intensity of stress was assessed by the change in the number of colony-forming units by the fourth hour of stress exposure relative to the moment of the onset of stress exposure (Table 1). Several levels of stress strength were distinguished: subinhibitory exposure (the number of CFUs in the stressed culture increased during the cultivation time); mild stress (inhibitory exposure, the number of CFUs in the culture remained at the same level as at the time

of stressor application); moderate stress (the number of CFUs decreased by about one order of magnitude) and severe stress (the number of CFU decreased by more than one order of magnitude).

TABLE 1

THE NUMBER OF COLONY-FORMING UNITS IN *E. COLI* CULTURE AFTER FOUR-HOUR EXPOSURE TO STRESSORS

Conditions	lgCFU/ml
Control, unstressed	8.3 ± 0.4*
30 mg/ml of NaCl	8.1 ± 0.3*
50 mg/ml of NaCl	7.6 ± 0.1
70 mg/ml of NaCl	6.9 ± 0.6
100 mg/ml of NaCl	6.1 ± 0.4*
200 mg/ml of NaCl	2.8 ± 1.9*
0.125 mg/ml of CH_3COOH	8.4 ± 0.5*
0.25 mg/ml of CH_3COOH	7.5 ± 0.4
0.5 mg/ml of CH_3COOH	7.3 ± 0.1
2 mg/ml of CH_3COOH	5.7 ± 1.2*
42 °C	8.2 ± 0.3*
45 °C	8.1 ± 0.2*
55 °C	0

Note. The number of CFU/ml at the time of stressor application was 7.4 ± 0.3; * – statistically significant difference from that at the time of stressor application ($N \geq 3$; T-test; $p \leq 0.050$).

Subinhibitory exposure had no effect on the expression level of the *soxRS* regulon genes (data not shown). In response to exposure of 50–100 mg/ml sodium chloride (mild and moderate stress), the level of *soxS* gene expression increased in *E. coli* cells in a dose-response manner; more intense osmotic stress did not induce changes in gene expression (Fig. 1b).

Under mild osmotic stress, the change in expression occurred in two stages: the gene expression level decreased after an increase in the initial stage of sodium chloride exposure and then began to increase again after the third hour of cultivation. An increase in *soxS* gene expression after addition of acetic acid to the concentrations that did not reduce the number of CFUs in the culture (0.25–0.5 mg/ml) was observed in the first 15 min from the onset of exposure; more intense acid stress was accompanied by a decrease in gene expression (Fig. 1g). The expression of *soxS* was lower in cells subjected to heating compared to cells grown under optimal conditions (37 °C) regardless of the severity of heat stress (Fig. 1e).

Changes in *nfo* gene expression under stress factors were similar to changes in *soxS* gene expression: mild and moderate osmotic shock caused an increase in gene expression, acid shock, which did not decrease the number of CFUs, slightly increased gene expression (Fig. 2), and more severe acid stress and heat exposure led to a decrease in gene expression (data not shown).

Therefore, under the conditions studied, activation of *soxRS* regulon gene expression was induced by exposure to sodium chloride and, to a lesser extent, acetic acid, while heat shock was accompanied by a decrease in gene expression. An increase in the expression level was observed in cells subjected to mild and moderate stresses, while severe stresses, which caused the death of a significant number of bacterial culture cells regardless of their physicochemical nature, were accompanied by a decrease in *soxRS* regulon gene expression. A decrease in gene fusion expression does not appear to be a specific response, but rather a consequence of a general metabolic suppression and inhibition of protein synthesis, including the reporter β-galactosidase.

The data obtained are consistent with the results of transcriptome analysis, which demonstrated an increase in the expression of the *soxRS* regulon genes (*soxS*, *fumC*, *fpr*, *acnA*) in *E. coli* cells when exposed to 0.3 M (17.5 mg/ml) sodium chloride [24]. Activation of *soxS* gene expression was also observed in *E. coli* cells subjected to osmotic shock induced by exposure to 0.4 and 0.9 M sucrose [25].

An increase in *sodA* mRNA synthesis in *Bacillus cereus* cells grown in media with pH = 5.4–4.5 and an increase in superoxide dismutase activity in *Staphylococcus aureus* cells grown in medium with pH = 4.0 and pH = 2.0, compared to cultivation in medium with neutral pH, have been previously shown [26, 27], suggesting activation of the *soxRS* regulon under conditions of acid stress. In this study, we demonstrated a slight increase in *soxRS* regulon gene expression during the initial stages of development of acid stress induced by acetic acid exposure.

Our results showed a decrease in the level of gene expression in cells grown at temperatures higher than optimal (37 °C). Earlier studies showed an increased level of *soxS* gene expression in cells grown at 43 °C compared to cells grown at 30 °C, which is regarded as an activation of expression in response to heat [24]. On the other hand, decreasing the cultivation temperature relative to the optimal level could cause a decrease in gene expression, which could also explain the observed differences in *soxS* expression level.

Thus, only osmotic stress induced by sodium chloride application, out of the three stress conditions investigated (exposure to acetic acid, sodium chloride, or heating), was accompanied by a significant activation of *soxRS*-regu-

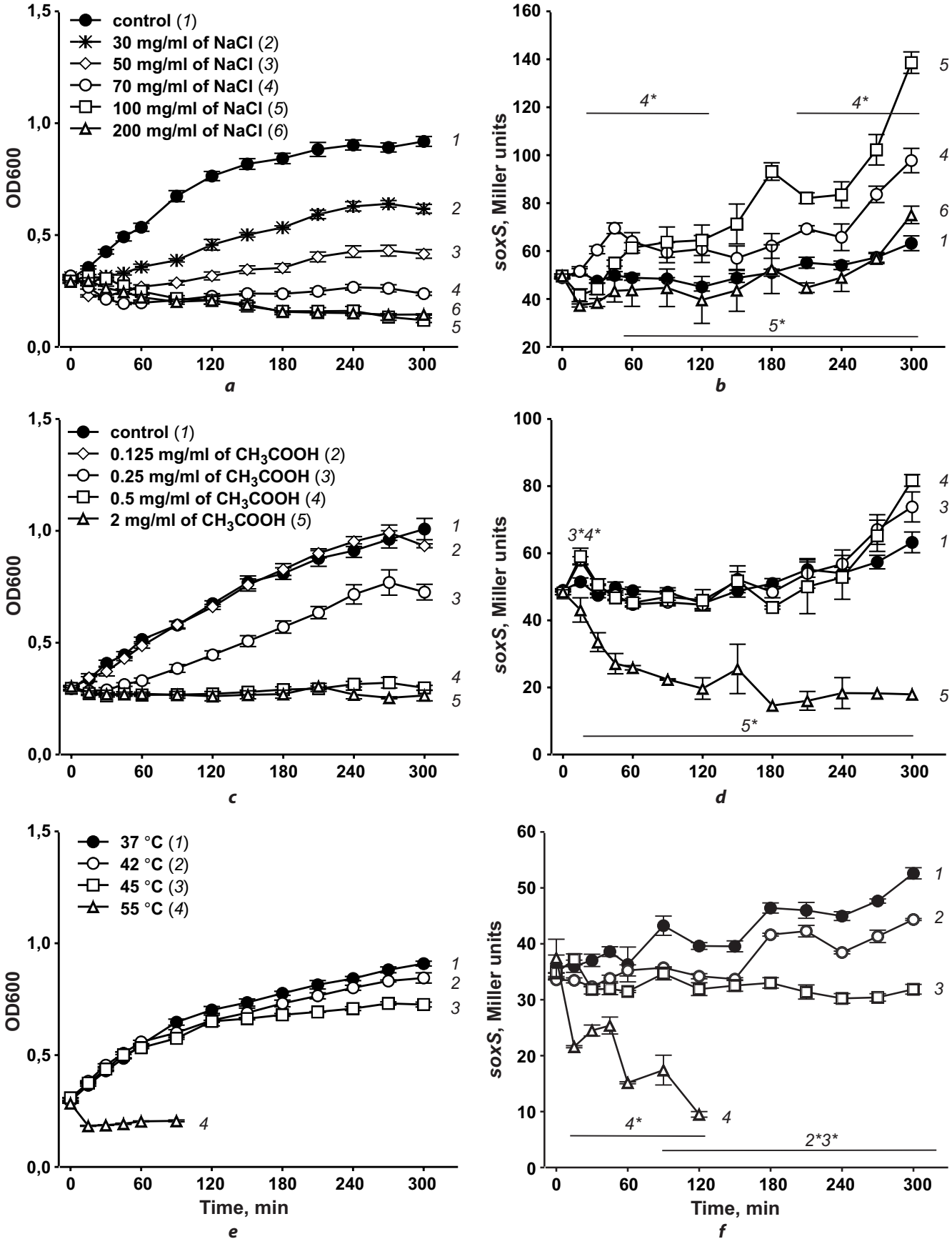

FIG. 1.

*Changes in the optical density (OD600) of E. coli culture and soxS gene expression in E. coli EH40 cells in response to osmotic (**a**, **b**), acid (**c**, **d**), and heat stress (**e**, **f**): * – statistically significant difference from the unstressed culture (control (1)) (N ≥ 3, T-test; p ≤ 0.050)*

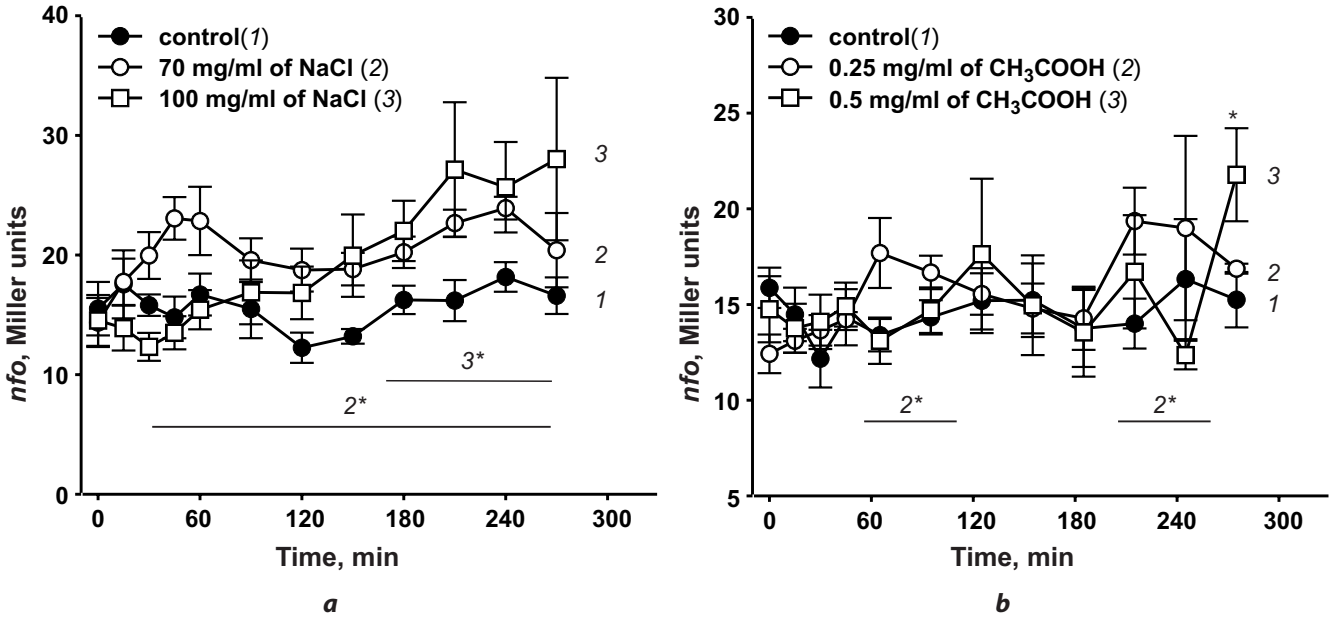

FIG. 2.

*Changes in nfo gene expression in E. coli N9213 cells in response to osmotic (**a**) and acid (**b**) stress: * – statistically significant difference from the unstressed culture (control (1)) (N ≥ 3; T-test; p ≤ 0.050)*

lon genes of antioxidant defence. Sublethal exposure to sodium chloride, causing a 2–2.5-fold increase in the expression of *soxRS* regulon genes, may provide pre-adaptation of bacteria to the factors that this regulon is aimed at counteracting, including antibacterial drugs.

Financing

This study was financially supported by the Ministry of Science and Higher Education of the Russian Federation (AAAA-A19-119112290009-1).

Conflict of interest

The authors of this article declare the absence of a conflict of interest.

The studies were conducted without the use of animals and without using humans as test subjects.

Acknowledgements.

The authors express their sincere gratitude to Prof. B. Demple and Prof. R.G. Martin for providing the bacterial strains.

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