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EXPERIMENTAL ARTICLES

Assessment of Iron Toxicity Using a Luminescent Bacterial Test with an *Escherichia coli* Recombinant Strain

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Abstract—The toxic effect of the Fe^{2+} and Fe^{3+} ions on the luminescent recombinant *Escherichia coli* strain with the *luxCDABE* operon was studied in short- and long-term experiments. At 30-min exposure of bacteria to the iron ions, the effective concentrations of Fe^{2+} and Fe^{3+} resulting in acute toxicity (EC_{50}) were 8.5 and 1.3 mg/L, respectively. In the long-term (24 h) experiment, during active bacterial growth, the toxicity index for Fe^{2+} and Fe^{3+} was 65.5 and 62.8, respectively. Addition of the iron ions into the medium did not suppress growth, although it inhibited luminescence. Comparative analysis of the short- and long-term experiments made it possible to assess iron toxicity at the concentrations from 0.5 to 20 mg/L (as calculated for the Fe^{2+} and Fe^{3+} ions). Iron ions were found to affect only the reactions that were not vitally important for the cell. At the same time, they had no negative effect on the genetic mechanisms and protein synthesis, thus indicating non-specific toxicity of Fe^{2+} and Fe^{3+}.

Keywords: bioluminescence, *Escherichia coli* recombinant strain, toxicity, bacterial growth

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In bacterial cells, iron is known to be present as two ions, Fe^{2+} and Fe^{3+}, which may be converted to one another. Both ions participate in a number of biological reactions including those associated with electron transfer, heme biosynthesis, and enzymatic activity [1]. However, while iron is important for the biological systems, its concentration in the cells should be maintained at a definite low level. At high concentrations iron has a toxic effect. The iron cations generate reactive oxygen species, which cause peroxidation of the cell membrane lipids, as well as protein and DNA damage [2].

Marine luminescent bacteria are widely used in the rapid tests, which make it possible to elucidate the integral toxicity of chemical substances and their mixtures [3]. The addition of a chemical compound alters bacterial metabolism, thus affecting the luciferase activity and, as a consequence, changing the luminescent intensity. The effect of a toxicant on the luminescent bacteria correlates with that on the higher plants and human tissue cultures [3, 4]. To determine the type of toxic effect of chemical compounds, two modifications of bacterial luminescence test are used: in the short-term experiments (30 min) non-specific toxicity is assessed, while in the long-term experiments with actively growing bacteria, specific toxicity of a chemical compounds affecting protein synthesis and genetic structure may be revealed [5, 6]. It was shown that a number of compounds possessed low toxicity at 30-min exposure, while in long-term experiments their active concentration decreased more than 1000-fold [7].

We modified the luminescent analysis and used a recombinant *E. coli* strain with the insertion of the complete *lux* operon as the test-object. This modification retains the advantages of the method. However, unlike marine luminescent bacteria, the recombinant *E. coli* strain does not require sodium chloride, which may affect the toxicity indexes [8, 9].

An effect of iron on the luminescence intensity and growth of luminescent bacteria, as well as on the native and recombinant *E. coli* strains has been described in literature. Optimal growth of *E. coli* was observed at iron concentrations of 0.1–2.0 mg/L [10, 11]. Addition of 2.8 mg/L of the Fe^{2+} ions was shown to cause a decrease in *E. coli* growth, and the concentration of 29 mg/L resulted in almost complete growth inhibition [11]. Addition of Fe^{2+} at concentrations from 0.56 μg/L to 56 mg/L to *Photobacterium phosphoreum* cells grown in a synthetic medium did not affect the growth of the culture during the first 9 h: the number of cells in the experimental and control samples was the same [12]. The data regarding the effect of iron on luminescence are contradictory. Fe^{3+} in concentration of 56 mg/L caused a slight decrease of bacterial luminescence [13]. A 50% suppression of *Vibrio fischeri* bioluminescence in the Microtox commercial preparation was observed 15 min at 7 mg/L Fe^{3+} [14]. The effect of Fe^{2+} on the commercial preparation Ecolum

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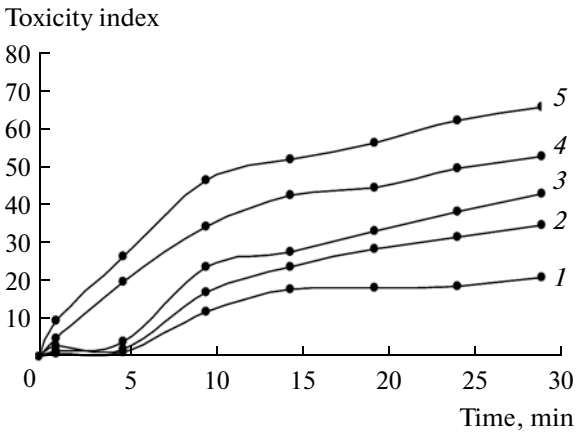

Fig. 1. Dependence of toxicity index depending on the time of incubation of *E. coli* with the Fe^{2+} ions and on their concentration in the short-term experiment. Concentrations of the Fe^{2+}, mg/L: 1—the curve (*1*), 2—the curve (*2*), 5—the curve (*3*), 10—the curve (*4*), and 20—the curve (*5*).

(prepared on the base of a recombinant *E. coli* strain) in the short-term experiment was described: 180 mg/L Fe^{2+} caused a 50% decrease in *E. coli* bioluminescence [15]. The same concentrations activated the luminescence of the marine bacteria *P. phosphoreum* [15]. Therefore, conflicting results of different authors indicate a non-specific toxic effect of iron at short-term exposure.

The goal of the present work was to elucidate the characteristics of toxic action of iron both in short-term experiments in real-time mode and in long-term experiments for the assessment of its possible specific toxicity towards luminescent bacteria.

MATERIALS AND METHODS

Subjects of the study for short-term experiments. The bacterial test Ecolum (Immunotech, Russia) developed in the laboratory of biologically active compounds of the Moscow State University was used in the experiments. The test system contained freeze-dried *E. coli* K12 TG1(pF1) cells with the insertion of the complete *CDABE lux* operon from the luminescent system of *V. fischeri* 6MSU [16].

Bacterial preparations for short-term experiments. Sterile distilled water (10 mL, pH 7.0) was added to the vial containing freeze-dried bacteria for the rehydration of the cells. The obtained suspension was diluted with water to the cell concentration in the suspension of $(2–3) \times 10^8$ cells/mL [16]. For assessment of the effect of the medium, the initial suspension was centrifuged at 6000 *g* for 15 min. The pellet was then resuspended in distilled water. The final concentration of the cells in the sample was $(1.5–2) \times 10^7$ cells/mL.

Bacterial preparations for long-term experiments. *E. coli* TG1 cells grown in LB medium were used in the experiments [17]. Cultivation was carried out under shaking (180–200 rpm) at 27°C for 8–9 h in 750-mL flasks containing 100 mL of the medium. The culture was then dispensed in 1.5-mL portions into 10-mL vials containing the toxicant; the cell density was $(1.5–2) \times 10^4$ cells/mL. The salts of $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$ in the range of concentrations of 0.5–20 mg/L Fe^{2+} or Fe^{3+} ions) were used as toxicants.

The experiments were performed under stationary conditions at 25–26°C for 24 h. Every two hours, bacterial growth and luminescence intensity were determined in the vials [7].

Measurement of bacterial luminescence. For the measurement of luminescence intensity, the sample was prepared containing the following: (1) in the short-term experiments, 0.1 mL of the Ecolum bacterial suspension and 0.9 mL of distilled water or the same volume of a toxicant; (2) in the long-term experiments, 0.1 mL of the cell suspension and 0.9 mL of distilled water [15, 16].

Bacterial luminescence intensity was measured using a Luminometer 1251 BioOrbit (Finland) and "Biotox-10" (Russia) for 30 min in the short-term experiment, as well as in the samples that were collected every two hours during the chronic experiment.

Toxicity index determination. To determine toxicity index, parallel measurements of bioluminescence in the control (without toxicants) and experimental samples were performed. The data were obtained from three repetitions. The error of all measurements did not exceed 10%.

Toxicity index was calculated using the following formula: $T = 100 \times (I_0 - I)/I_0$, where I_0 and I are luminescence intensities at a fixed exposure time in the control and experiment, respectively.

The universally adopted toxicologcal indexes of effective concentrations EC_{20} and EC_{50} were used. EC_{20} results in 20% inhibition of luminescence, and the sample is considered toxic at this concentration. EC_{50} results in 50% inhibition of bacterial luminescence, and at this concentration its acute toxicity is declared [4].

EC_{50} was calculated according to the Γ- function using the formula $\Gamma = 100 \times (I_0 - I_t)$, where I_0 and I_t are the luminescent intensities in the absence and in the presence of a toxicant, respectively [4, 8].

Cell growth. The number of bacterial cells was determined by optical density of the suspension at $\lambda =$ 590 nm using a KF77 photoelectrocolorymeter (Poland) and calculated using the calibration curve.

$FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$ were of the chemically pure and pure for analysis grade; for culture media the reagents from Difco were used.

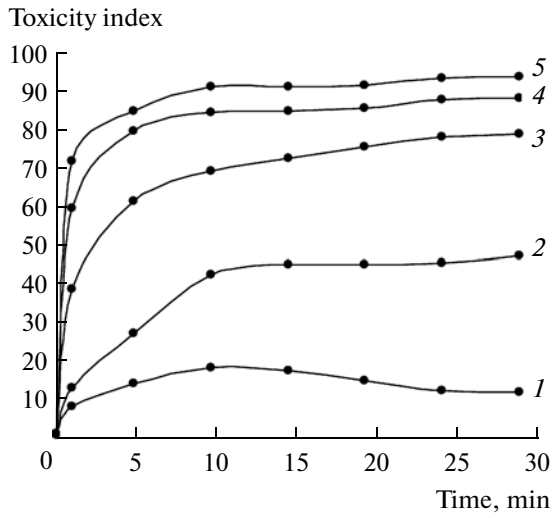

Fig. 2. Dependence of toxicity index depending on the time of incubation of *E. coli* with the Fe^{3+} ions in the short-term experiment. Concentrations of the Fe^{3+}, mg/L: 0.1—the curve (*1*), 0.5—the curve (*2*), 1—the curve (*3*), 2—the curve (*4*), and 5—the curve (*5*).

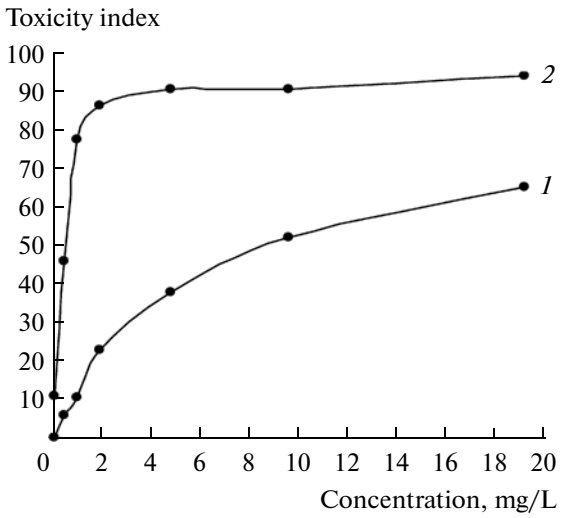

Fig. 3. Dependence of toxicity index depending on the concentrations of the Fe^{2+} and Fe^{3+} in the a short-term experiment with 30-min exposure: 1—Fe^{2+} (*1*) and, 2—Fe^{3+} (*2*).

RESULTS AND DISCUSSION

Assessment of the effect of Fe^{2+} and Fe^{3+} on bioluminescence of the recombinant *E. coli* strain at the short-term exposure (30 min) demonstrated a decrease in the luminescence intensity at concentrations of 0.1–20 mg/L (Figs. 1, 2).

Analysis of the experimental results revealed differences in the dynamics of toxicity index of the luminescent bacteria affecting the presence of Fe^{2+} (Fig. 1) and Fe^{3+} (Fig. 2). In the case of Fe^{2+}, the toxicity index increased gradually throughout the period of observation (Fig. 1), while in case of Fe^{3+} (Fig. 2) the toxicity index did not change significantly during the first 10 min. Fe^{3+} probably interacts with the cell targets more rapidly than Fe^{2+}. The degree of the process depended on the ions concentration (Fig. 3). The EC_{20} and EC_{50} values for both types of the iron ions were determined. For Fe^{2+}, EC_{20} = 1.3 and EC_{50} = 8.5 mg/L; for Fe^{3+}, EC_{20} = 0.05 and EC_{50} = 1.2 mg/L. Thus, the Fe^{3+} ions were more toxic for *E. coli* cells than the Fe^{2+} ions.

Components of the culture medium can bind the metal ions, affecting the toxicity assessment. We made an attempt to remove lyophilization medium. For the washed cells, EC_{50} was 1.2 and 0.5 mg/L for Fe^{2+} and Fe^{3+}, respectively. This indicates that the presence of the medium in the samples significantly decreased the effective concentration of the affecting substance. When the cells were washed from the medium, sensitivity of the method increased tenfold for Fe^{2+} and 2.5-fold for Fe^{3+}, which exhibited high toxicity under the standard experimental conditions.

Our results regarding iron toxicity differ from those obtained previously in short-term experiments [11–15]. These differences may be due both to the application of different test systems and to modifications of the method itself: other researchers could operate with different numbers of the cells. The sensitivity of the method is known to depend on the number of cells in the sample, which affects the range of active concentrations of the toxicant. Deryabin and Karimov, in their work [15] with luminescent *E. coli* cells from the Ecolum kit, used concentration of 10^9 cells/mL (two orders of magnitude higher than in our study), and EC_{50} for the Fe^{2+} was 180 mg/L.

Nowadays new methodical approaches are developed for the application of luminescent bacteria in the evaluation of toxicity of compounds whose negative effect manifests itself later in time if the cells of the test culture are actively growing in a rich medium. During prolonged exposure to the test object, the toxic compounds may suppress protein and DNA synthesis leading to suppression of cell division [2, 18]. Based on the results of the long-term experiment with *E. coli* grown for 24 h in the medium containing the iron ions at concentrations of 0.5–10 mg/L, iron toxicity indexes were calculated and the effect of iron on bacterial growth was analyzed.

An initial point for comparison of the indexes of acute and chronic iron toxicity in the short- and long-term experiments were the values measured at the 30th minute of incubation. In the long-term experiment, the iron toxicity index values were lower at the same concentrations of the Fe^{2+} and Fe^{3+} (Fig. 3) than those obtained in the short-term experiment (Figs. 4, 5). These data indicate that the medium used

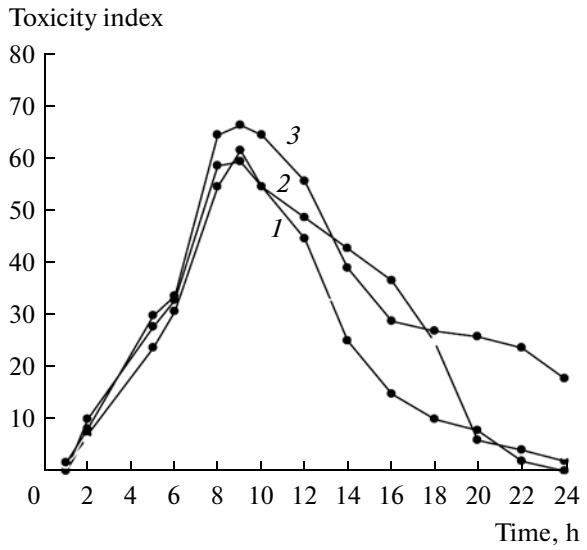

Fig. 4. Dependence of the Fe^{2+} toxicity index depending on the concentration of the ions and on the time of incubation with *E. coli* cells in the a long-term experiment. Concentrations of the Fe^{2+}, mg/L: 0.5—the curve (*1*), 1—the curve (*2*), and 10—the curve (*3*).

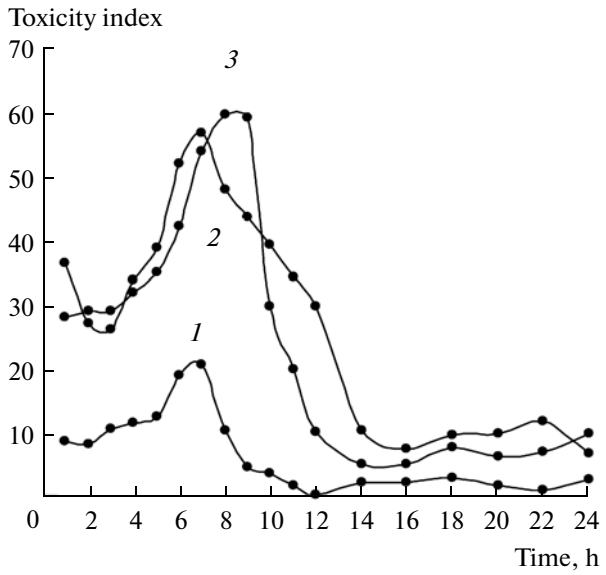

Fig. 5. Dependence of the Fe^{3+} toxicity index depending on the concentration of the ions and on the time of incubation with *E. coli* cells in the a long-term experiment. Concentrations of the Fe^{3+}, mg/L: 0.5—the curve (*1*), 1—the curve (*2*), and 10—the curve (*3*).

in chronic experiments is even richer than the lyophilization medium of the Ecolum biotest (unwashed cells). It contains more organic substances able to bind with iron ions, thus decreasing their effective concentration [7].

Results of the long-term experiment indicated that the toxic effect of Fe^{2+} in the range of concentrations of 0.5–10 mg/L was maximal at 7–12 h of bacterial growth: toxicity index for Fe^{2+} reached its maximum (65.5) during this period and then decreased to 18.4 after 24 h of growth. Fe^{2+} toxicity index did not depend on the iron concentration in the chosen range (Fig. 4). It may be suggested that at a lower Fe^{2+} concentration (0.5 mg/L) the saturation of the iron-binding centers occurs. The absence of additivity in bacterial cell responses to the effect of some metals has been discussed in the literature. While no suppression of the physiological cell processes occurs within a certain range of their concentrations (the so-called hormesis effect), the mechanisms of this effect are still unclear [19].

Fig. 5 shows the dynamics of Fe^{3+} toxicity in a long-term experiment. The toxicity index increased during the first hours of cultivation and reached its maximum at 11 h of growth. The maximal value of toxicity index at Fe^{3+} concentrations of 1–10 mg/L was 62.8; it subsequently decreased and reached the value of 20–10. At the Fe^{3+} concentration of 0.5 mg/L, the curve of toxicity was of the same form,

Effect of the Fe^{2+} and Fe^{3+} on the *E. coli* growth in the long-term experiment

Time of cultivation, h	Number of cells, 10^9/mL				
	Control	Fe^{2+}		Fe^{3+}	
		1 mg/L	20 mg/L	0.5 mg/L	10 mg/L
12	0.8 ± 0.03	0.8 ± 0.03	0.8 ± 0.03	0.8 ± 0.03	0.8 ± 0.03
14	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02
16	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.02
18	1.0 ± 0.03	1.0 ± 0.03	1.0 ± 0.2	1.0 ± 0.02	1.0 ± 0.03
20	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
22	2.0 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	2.0 ± 0.2
24	4.4 ± 0.3	4.4 ± 0.3	4.4 ± 0.3	4.4 ± 0.3	4.4 ± 0.3

although the toxicity index was significantly lower (its maximum being 20.7).

Comparative study of the data obtained in the standard (30 min) and long-term tests demonstrated that maximal toxicity of Fe^{2+} after 10 h of growth and that of Fe^{3+} after 11 h were almost equal to the toxicity determined by the luminescent method in the short-term experiment.

Effect of the iron ions on bacterial growth was studied using the same range of concentrations of the ions (0.5–20 mg/L) that have been used in the measurements of *E. coli* bioluminescence and growth (table). In the control and experimental samples, bacteria grew, reaching the cell density of 10^4–10^9 cells/mL. The table shows the results of experiments in the presence of high (20 mg/L) and low (1 mg/L) concentrations of the Fe^{2+} and Fe^{3+}. Cell growth in the experiment and in the control was equal and did not depend on the presence of iron ions. The metabolic processes affecting the rate of cell division were probably not affected [6]. However, bioluminescence inhibition reflects a toxic action of the iron ions on the metabolic activity of bacteria.

Our results on the Fe^{2+} and Fe^{3+} toxicity and on bacterial growth could be explained using the data reported by other authors. Firstly, Fe^{2+} and Fe^{3+} may be subjected to hydrolysis and precipitate as $Fe(OH)_3$. This process misrepresents the real concentration of the ions. Secondly, an effect of iron on *E. coli* cells is known to enhance the oxidative stress and to increase the level of reactive oxygen species in the cells [2, 13]. In such cases, the adaptive mechanisms of stress response regulation are activated in bacterial cells, and the bacteria become resistant and may survive in the presence of the toxicant (the iron ions) [20]. Thirdly, activation of the oxidation processes induced by the addition of iron leads to peroxidation of the lipids, which results in the formation of an aliphatic aldehyde. The latter is a substrate for bacterial luciferase, which enhances the enzyme activity, thus altering the level of luminescence as the index of toxicity. Divalent iron was shown to have no direct effect on luciferase [21].

Thus, the conclusions are the following. Short-term experiments demonstrated that Fe^{2+} and Fe^{3+} differently affected bacterial cells. The rate of interaction of the cells with Fe^{3+} appeared to be much higher (the effect was visible after one minute of exposure), as did the toxicity index, which reached its maximum after 10 min of incubation. Thus, the biological activity of the Fe^{3+} ions is higher than that of Fe^{2+}. In long-term experiments, the maximal toxicity index of Fe^{2+} was the same as in the short-term experiments, while that of Fe^{3+} was significantly lower.

Application of the approaches based on cultivation of the cells with a toxicant under conditions of active bacterial growth in combination with the short-term bioluminescent method detecting nonspecific toxicity made it possible to assess the real character of the iron toxicity.

In general, the results of the standard experiment with 30-min exposure of the test organism to the toxicant, together with the results of the long-term experiment (up to 24 h), gave a more reliable evaluation of toxicity of both types of the iron ions. Bioluminescence inhibition which did not lead to the changes in the rate of cell division at the studied iron concentrations allowed us to make a conclusion regarding the nonspecific toxicity of iron; it does not affect the genetic apparatus and protein and nucleic acid synthesis.

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