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EXPERIMENTAL ARTICLES

Induction of the Unculturable State in *Escherichia coli* K12 with 2,4,6-Trinitrotoluene

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Abstract—The toxic effect of high (200 mg/l) 2,4,6-trinitrotoluene (TNT) concentrations on *Escherichia coli* K12 cells in the absence of exogenous nutrient sources (incubation in 0.5% NaCl) was shown to manifest itself in the transfer of the culture to an unculturable but viable state; its reversal depends on the duration of culture contact with the xenobiotic and the conditions of cell recultivation. The likelihood that cell succession to death forms the basis of the physiologo–biochemical mechanism of the unculturable state in *Escherichia coli* K12 population under conditions of combined toxic and starvation stress is discussed.

Key words: 2,4,6-trinitrotoluene (TNT), toxicity, *Escherichia coli* K12, unculturable state, cell succession to death.

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We have established earlier that, in the course of elimination of high 2,4,6-trinitrotoluene (TNT) concentrations (200 mg/l), *Escherichia coli* cells were exposed the toxic effect of this xenobiotic. The toxic effect was manifested in reduced cell size, the appearance of dwarf coccoid forms, and increased refractive index (refractivity) [1]. Along with a change in the cell size and morphology, the redox processes in the cells were inhibited: the glucose utilization rate, the levels of $NAD \cdot H_2$ ($NADP \cdot H_2$) and oxidized flavin cofactors, and the membrane cell potential decreased and the population energy profile changed [2]. The changes revealed are characteristic of the transfer of vegetative cells to an unculturable state (US) [3–6]. Thus, it is obvious that the toxic stress caused by high TNT concentrations promoted transfer of *E. coli* K12 to the US. However, despite this fact, the culture appeared to be capable of eliminating up to 90% of the xenobiotic under the conditions of batch cultivation on the medium containing glucose as a source of carbon and energy and ammonium nitrogen as a source of nitrogen. After elimination of the main amount of TNT, the cell population reverted to vegetative cells [1, 2].

The aim of the study was to establish the possibility of *E. coli* K12 cell transfer to the unculturable state and to determine conditions sufficient for such a transfer and its reversal.

MATERIALS AND METHODS

The subject of the study was the gram-negative strain *Escherichia coli* K12 from the collection of the Department of Microbiology, Kazan State Medical Academy. The inoculum was grown in 100-ml flasks on nutrient broth for 16–18 h at 30°C under forced aeration conditions and introduced into the medium at a concentration of 6.4×10^7 cells/ml. The incubation was carried out in 0.5% NaCl with the addition of TNT (0.2 g/l).

The biomass concentration was determined by counting the total cell number using a Thoma–Goryaev counting chamber.

In order to determine the cell capacity for colony formation, appropriate dilutions of the cell suspension in 5–7 replicates were plated on petri dishes with nutrient agar (NA); the plates were incubated at 28°C for 72 h, and the appearance of colonies was controlled every 24 h. The colonies were counted in dilution variants producing no more than 80 colonies per plate. The results were expressed as averages and standard deviations.

The Kogure test [7] was used to determine the number of living (culturable) and viable, but unculturable, cells. The test is based on the assumption that both living and viable cells (i.e., those not forming colonies on solid nutrient media) increase in size on addition of a nutrient substrate in the presence of nalidixic acid that blocks division. Enlarged viable cells are easily differentiated from small-sized, unresponsive cells.

The number of cells with damaged membranes (dead cells) was estimated by staining with propidium iodide (PI). Propidium iodide was added to the cell suspension to the final concentration of 2.9 μM. After 10-min incubation, the samples were centrifuged and washed twice with 0.9% NaCl. Fluorescence was measured using a SIGNE-4M spectrofluorimeter with a

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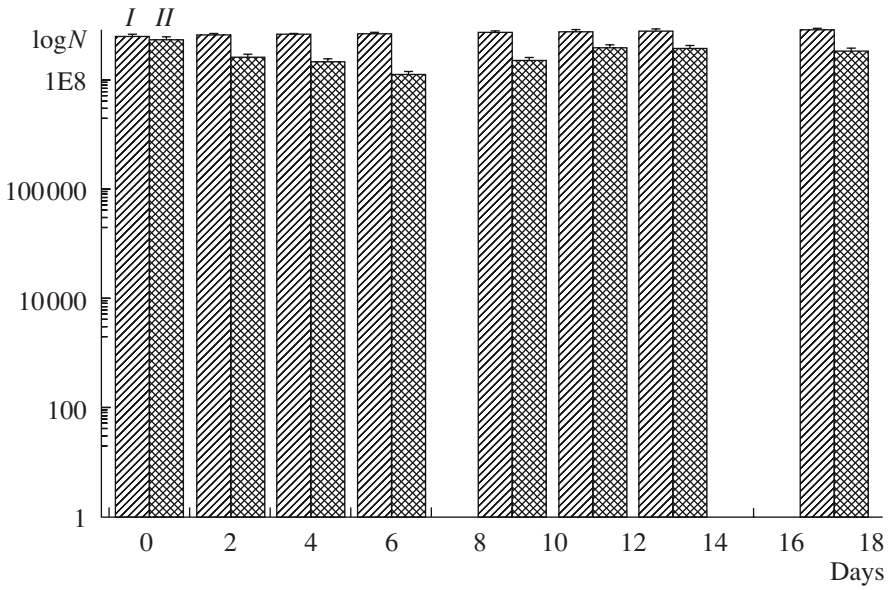

Fig. 1. Change in the total cell number (*I*) and the number of colony-forming units (*II*) in *E. coli* K12 cell suspension incubated in 0.5% NaCl.

10-nm slit width in the excitation channel (495 nm) and in the emission channel (615 nm). The results were standardized by optical density at 670 nm and expressed as a percentage in relation to the sample used as the control [8].

The effectiveness of TNT transformation was assessed by the TNT concentration decrease in the culture fluid. The method based on the color reaction between TNT and Na_2SO_3 under alkaline conditions was used for TNT determination [9].

The species identity of unculturable cells after resuscitation was assessed on the basis of their biochemical properties using the IEMS-Reader plate photometer (Finland) according to the manufacturer's instruction.

Mathematical data processing was carried out using the standard Origin 6.1 software package. In the work, all the experiments were made in at least five replicates. The group of findings was considered homogeneous if the standard deviation σ did not exceed 13%. The intergroup differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

It is known that TNT cannot serve as a utilizable source of energy, carbon, and nitrogen. The transformation of the xenobiotic by bacteria is feasible under the cometabolism conditions [10]. The data presented by us earlier suggest that the use of glucose as an energy source for cell survival competes with the supply of reducing equivalents for the xenobiotic reduction system. The outcome of the competition is influenced by the xenobiotic concentration. At high TNT concentrations, the use of glucose for obtaining reducing equivalents of the reduction system predominates: the amount of the xenobiotic nitroreduction products increases, whereas the total amount and the number of colony-forming cells are retained at the initial inoculum level. With a decrease in the TNT concentration to 50 mg/l, the number of colony-forming cells begins to increase and the number of nitroreduction products remains at a level attained at high xenobiotic concentrations [1, 11]. These data lead us to believe that the transfer of *E. coli* cells to the unculturable state is most likely at high TNT concentrations and at concentrations of energy sources excluding their use for cell growth and multiplication. We tested this assumption by incubating *E. coli* K12 with TNT in 0.5% NaCl in the presence of only endogenous sources of energy and reducing equivalents.

As seen from Fig. 1, when *E. coli* K12 cells were incubated in 0.5% NaCl solution (the control variant), both the total cell number and the number of colony-forming units did not change significantly in 17 days of incubation.

The introduction of TNT at a concentration of 200 mg/l into the incubation medium did not result in a decrease of total cell numbers (Fig. 2). As in the control variant, the total cell number did not change significantly through the whole incubation period. However, on the second day of incubation, the number of colony-forming cells decreased by three orders of magnitude; from the ninth day no growth was observed on nutrient agar. It should be noted that the culture proved to be incapable of eliminating TNT from the incubation medium (Fig. 3). Thus, at day 17, $85 \pm 5\%$ of the initial xenobiotic concentration was revealed in the medium, whereas in the presence of exogenous nutrient sources,

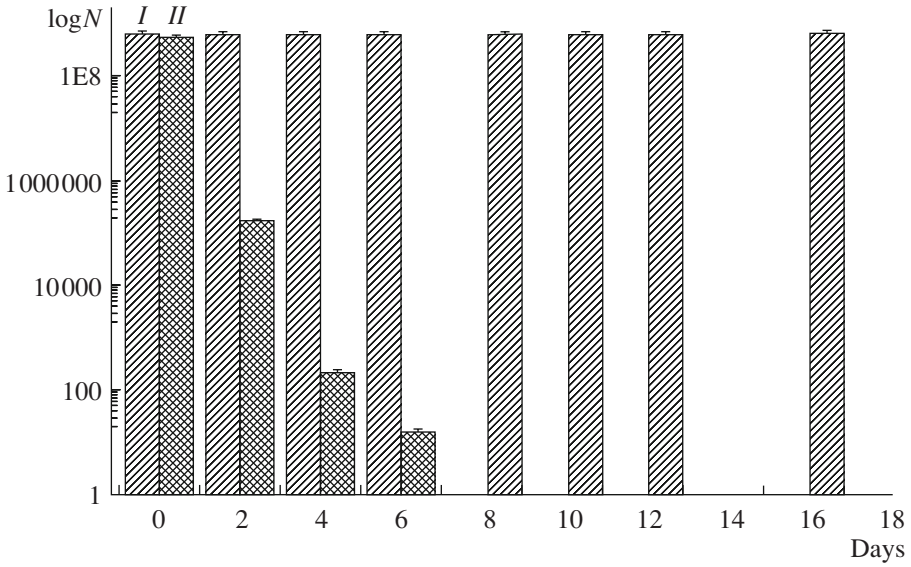

Fig. 2. Change in *E. coli* K12 total cell number (*I*) and the number of colony-forming units (*II*) for incubation in 0.5% NaCl with TNT (200 mg/l).

only 17 ± 7% of TNT remained in the medium after 8 h of cultivation [1]. It is noteworthy that a decrease in the xenobiotic concentration was observed only during the first nine days of incubation. This is most likely the result of a decrease in the metabolic activity of the cells being in contact with TNT.

As in the presence of exogenous nutrients [1, 2], a change in cell morphology was observed. They decreased in size. At the second day of incubation, the cells in contact with TNT were 1.00 ± 0.04 μm long, while the length of the control cells was 1.25 ± 0.09 μm. TNT-exposed cells lost motility almost completely, retaining only oscillatory movements. However, when *E. coli* K12 cells were incubated with TNT in 0.5% NaCl, we did not observe formation of coccoid bacterial cells [1].

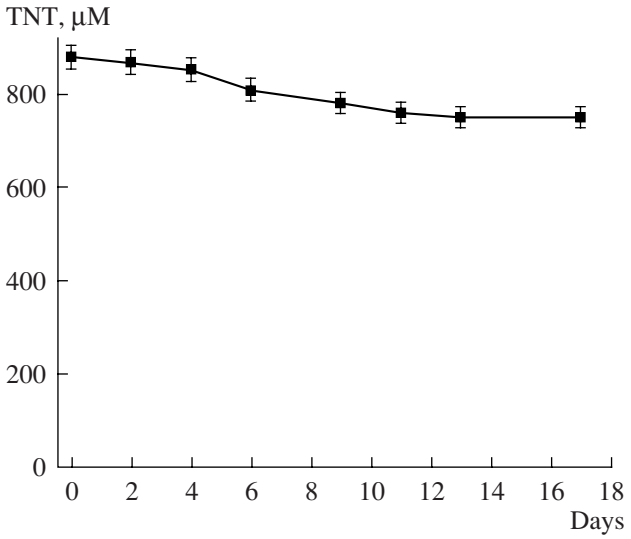

Fig. 3. Dynamics of TNT (200 mg/l) elimination from the medium (0.5% NaCl) where *E. coli* K12 were incubated.

To gain deeper insight into the character of changes in the populations of *E. coli* K12 cells, the Kogure test was performed, and the cells were stained with propidium iodide, the dye that stains dead cells [12].

As seen from the table, viable cells (increasing in size in the presence of yeast extract and nalidixic acid) were retained in the population for up to 17 days of observation. Their number decreased in the process of incubation, whereas the number of propidium iodide-permeable (dead) cells increased. After incubation with the xenobiotic for 11 and 17 days, the TNT-exposed cells were washed with 0.5% NaCl transferred to nutrient broth and nutrient broth with 0.025% yeast extract. Growth was noted only on nutrient broth with yeast extract inoculated with the cells of the culture incubated with TNT for 11 days. The cells grown after 16 h were compared with the control cells. They were identified as *E. coli* based on their cultural and biochemical properties.

At 17 days of cultivation with TNT, the number of large cells in the Kogure test decreased to 40%, and the number of PI-permeable cells increased to 57%. The cells of this population did not grow on nutrient broth with yeast extract, and they did not recover from the unculturable state. The comparison between the capacity for colony formation data presented in Fig. 2 and the data on the number of large cells in the table shows that the transfer of *E. coli* K12 cells into the unculturable state under conditions of their incubation with TNT in 0.5% NaCl occurred in the period between days 6 and 9.

The numbers of large (viable) cells determined by the Kogure test and of dead cells stained with propidium iodide at *E. coli* K12 incubation in 0.5% NaCl containing 200 mg/l of TNT

Time, days	Large (viable) cells, %		PI-permeable (dead) cells, %	
	Control	Experiment	Control	Experiment
9	95 ± 5	90 ± 4	2 ± 1	6 ± 3
11	92 ± 7	85 ± 9	7 ± 3	16 ± 3
17	89 ± 8	40 ± 7	11 ± 5	57 ± 10

Thus, on contact with TNT in the absence of exogenous sources of energy and renewable reducing equivalents, the cells of *E. coli* K12 transfer to the unculturable state; its reversal depends on the duration of the culture contact with the xenobiotic and the resuscitation conditions.

The absence of growth of the colonies of TNT-exposed cells on nutrient agar after the ninth day of incubation may be linked to both their transfer to the unculturable but viable state (adaptation of the cells to the cultivation conditions) and death. The analysis of changes in the number of living and dead *E. coli* K12 cells incubated in 0.5% NaCl in the presence of the xenobiotic suggests that degenerative processes underlie the physiological and biochemical mechanisms of the unculturable state of the population of TNT-exposed cells under conditions of combined starvation and toxic stress, and the changes we noted give evidence of succession of the cell population to death.

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