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EXPERIMENTAL ARTICLES

Induction of the SOS Response in *Escherichia coli* Cells under Osmotic Stress and in the Presence of N-Methyl-N'-Nitro-N-Nitrosoguanidine

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Abstract—We investigated the dynamics of the SOS response induction and the frequency of reversions induced by the monofunctional alkylating compound N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* cells exposed to osmotic stress for 1 h. During the stress treatment of the wild-type cultures adapted and not adapted to the alkylating agent, the maximum SOS response values and induced reversion frequencies were recorded twice. The SOS response values and induced reversion frequencies remained unchanged during the whole period after attaining the maximum values in adapted and nonadapted cells carrying a mutation in the excision repair gene. Presumably, the SOS mutagenesis mechanisms are turned on in the cells with an inactivated excision repair system earlier than in wild-type cells.

Key words: SOS response, adaptive repair, excision repair of nucleotides, SOS-dependent mutagenesis, N-methyl-N'-nitro-N-nitrosoguanidine.

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At the initial stage of research on DNA repair, several self-contained mechanisms involved in repairing DNA impaired by various deleterious factors were discerned. However, currently it is widely accepted that a global reparative network is in operation in the cells of living organisms. The mechanisms incorporated in the network overlap, and their activity varies depending not only on the mutagen, but also on the cultivation conditions and the physiological state of the cells involved. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-elicited DNA damage is ameliorated by direct methyl-group transfer from methylated DNA nitrogen bases to proteins with alkyl transferase activity. The two proteins Ada and Ogt remove methyl groups from the damaged bases in *E. coli* cells. Ada (O^6-methylguanine–DNA methyl transferase I) transfers alkyl groups to cysteine residues Cys-321 and Cys-69 located in the C- and N-terminal protein domains, respectively [1]. Cys-69-Alkylated Ada operates as the transcriptional activator of the adaptive response genes (*ada*+, *alk*A+, *alk*B +, and *aid*B+) and forms complexes with RNA polymerase, securing the binding of RNA polymerase to the transcription initiation site [2]. The adaptive response turns on in the cell after 60 min of cultivation with low concentrations of alkylating compounds [3]. O^6-methylguanine–DNA methyl transferase II (protein Ogt) is responsible for protecting the cell from the mutagenic effect of alkylating compounds prior to the optimum induction of the O^6-methylguanine–DNA methyl transferase I [4] (Fig. 1).

A key event in the process of adaptive response activation is the binding of methylated Ada to the σ^{70} or σ^{38} subunits of the RNA polymerase. The σ^{70} subunit, the product of the *rpo*S+ of *E. coli*, consists of 330 amino acid residues and is involved in the regulation of the adaptive response of the stationary phase cells [5]. Protein RpoS regulates the expression of the stationary phase genes and is involved in the cell's response to osmotic shock [6]. Although ^{me}Ada activates the transcription of the genes of the *ada* operon upon complexation both with σ^{70} and σ^{38}, a mutation in the *rpoS* gene decreases the expression of the *aid*B$^+$ even in the presence of ^{me}Ada. By contrast, ^{me}Ada inhibits σ^{38} binding to the promoter of the *alk*A$^+$ gene. Even though σ^{70} and σ^{38} recognize the same promoters, they differ in the pattern of their interaction with the promoters [7].

It is known that the mechanism of excision repair of nucleotides is implicated in restoring alkylating compound-damaged DNA. Some of the genes involved (*uvrA*$^+$, *uvrB*$^+$, and *uvrD*$^+$) contain SOS-dependent promoters [8, 9]. Proteins LexA and RecA regulate the expression of 20 to 30 genes of the SOS response [10]. The function of the SOS-response-induction signal is performed by single-stranded DNA regions, which are formed during the replication of the damaged DNA

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duplex. In the cells treated with electrophilic alkylating compounds, one of the two DNA strands may rupture at AP sites. They are formed due to the operation of 3-methyladenine DNA glucosidase II, the product of the *alk*A$^+$ gene that is involved both in the adaptive response and excision repair of bases [11]. DNA glucosylases, enzymes carrying out excision repair of DNA bases, excise the damaged base from the DNA strand while the sugar–phosphate backbone remains intact. This DNA site is termed an apurine/apyrimidine site (an AP site). The gap in the DNA is further increased by endo-, and, subsequently, exonucleases [12].

The binding of protein RecA to single-stranded DNA regions in the presence of nucleoside triphosphates reversibly converts it into the activated form. Activated RecA* promotes the autoproteolytic cleavage of protein LexA dimers located in the promoter regions of SOS genes, referred to as SOS boxes. This results in inducing the expression of these genes [10]. The mechanism of nucleotide excision repair is a precise DNA restoration mechanism. However, the expression of some SOS-dependent genes increases mutation frequency. Errors in the DNA resulting from the SOS response enhance the viability of cells in hypermutable populations under stress [13] (Fig. 1). Hence, the activation of a given DNA protection mechanism in a bacterial cell is contingent, apart from on the mutagen type involved, on the environmental conditions.

The goal of this work was to assess the dynamics of SOS response induction under osmotic stress in *E. coli* cells that were either adapted or unadapted to the monofunctional alkylating compound MNNG.

MATERIALS AND METHODS

This work used the following *E. coli* strains: B/r WP2 *trp*E65 and B/r WP2s *uvr*A, *trp*E65 obtained from the Vavilov Institute of General Genetics, Russian Academy of Sciences; and JC7623 *thr*-1, *ara*C14, *leu*B6, *lac*Y1, *gal*K2, *rpo*S396(Am), *arg*E3(Oc), *his*G4(Oc) and PJ2 *thr*-1, *ara*C14, *leu*B6, *lac*Y1, *gal*K2, *ada*2, *arg*E3(Oc), and *his*G4(Oc), obtained from the *E. coli* Genetic Stock Center (Yale University, USA). The revertant frequency was determined from the number of mutants reverting from tryptophan (strains B/r WP2 and B/r WP2s) and histidine (strains JC7623 and PJ2) auxotrophy to prototrophy (the reversion number). After cultivating *E. coli* cells overnight in LB medium (pH 7.4), the culture was diluted with fresh complete nutrient medium (1 : 20). The working culture was incubated in LB broth (pH 7.4) under aeration until it reached an OD of 0.4 (λ = 600 nm). The *E. coli* cells were thereupon centrifuged at 5000 *g* for 15 min. The pellet was washed twice and resuspended in potassium phosphate buffer (PPB), pH 7.4. To obtain MNNG-adapted cells, the medium was supplemented with the alkylating compound at a concentration of 2 μg/ml and incubated for 60 min. The suspensions of both adapted and unadapted cells were divided into eight parts: (i) the control cell suspension lacking NaCl and incubated in PPB for 60 min; (ii) the cell suspension containing NaCl (500 mM) and incubated in PPB for 60 min; and (iii–viii) the cell suspensions incubated in PPB with NaCl (500 mM) for 10, 20, 30, 40, 50, and 60 min before addition of MNNG. The cell subcultures were then centrifuged at 5000 g for 15 min, concentrated tenfold in PPB, and treated with MNNG at a final concentration of 10 μg/ml. After a 60-min incubation at 37°C, the cells were centrifuged, resuspended in fresh cold PPB, and divided into two parts. One of the parts was used to determine the number of viable cells by plating on LB agar. The other part was used to determine the number of prototrophic revertants on glucose mineral agar containing either tryptophan and thiamine at final concentrations of 1 and 0.5 μg/ml, or arginine, leucine (200 μg/ml), and histidine (8 μg/ml). The reversion frequency was estimated using the formula [14, 15] $M = a - b/g$, where M is the induced mutation frequency per viable cell, a is the revertant number (mean colony number for three petri dishes with glucose mineral agar supplemented with amino acids) obtained by inoculating 0.1 ml of the mutagen-treated cell suspension, b is the revertant number obtained by inoculating 0.1 ml of the mutagen-untreated cell suspension on petri dishes with glucose mineral agar without amino acids, and g is the viable cell number in 0.1 ml of the mutagen-treated cell suspension.

To monitor SOS response induction by means of the bioluminescent assay, the pPLS-1 p*cda*::*lux*CDABE, Am^R plasmid [16] isolated from *E. coli* C600 *F*$^-$, *thi*-1, *thr*-1, *leuB6, lacY1, tonA21, supE44* [17] was introduced into the tested strains via transformation. SOS response induction was determined from the induction of the SOS-dependent promoter of the *cda* gene that codes for colicine ColD. The bioluminescent assay was based on the method suggested by L.R. Ptitsyn [16]. Cells were incubated as described above, but PPB was replaced by LB broth with ampicillin (50 μg/ml). The cell culture aliquots (200 μg) were then cooled to room temperature for 10 min on a shaker (180 rpm). Bioluminescence was measured with a Biotox-6 device [16]. In these experiments, the expression level of the reporter *pcda::luxCDABE* genes was estimated from the bioluminescence induction factor (F_i). It was determined from the ratio of light emission of the mutagen-treated cell suspension ($luxA_i$) to that of the mutagen-untreated cell suspension ($luxA_o$): $F_i = luxA_i \times B_o/luxA_o \times B_i$. We analyzed the results taking into account the viable cell numbers in the mutagen-untreated (B_o) and mutagen-treated (B_i) cell suspensions.

Three repeats of each of the experiments were done. The data obtained were evaluated using Student's t test; the significance level P was 0.05 or below [18].

RESULTS AND DISCUSSION

A stress factor can result in the hypermutable state of bacterial cells that requires SOS response induction

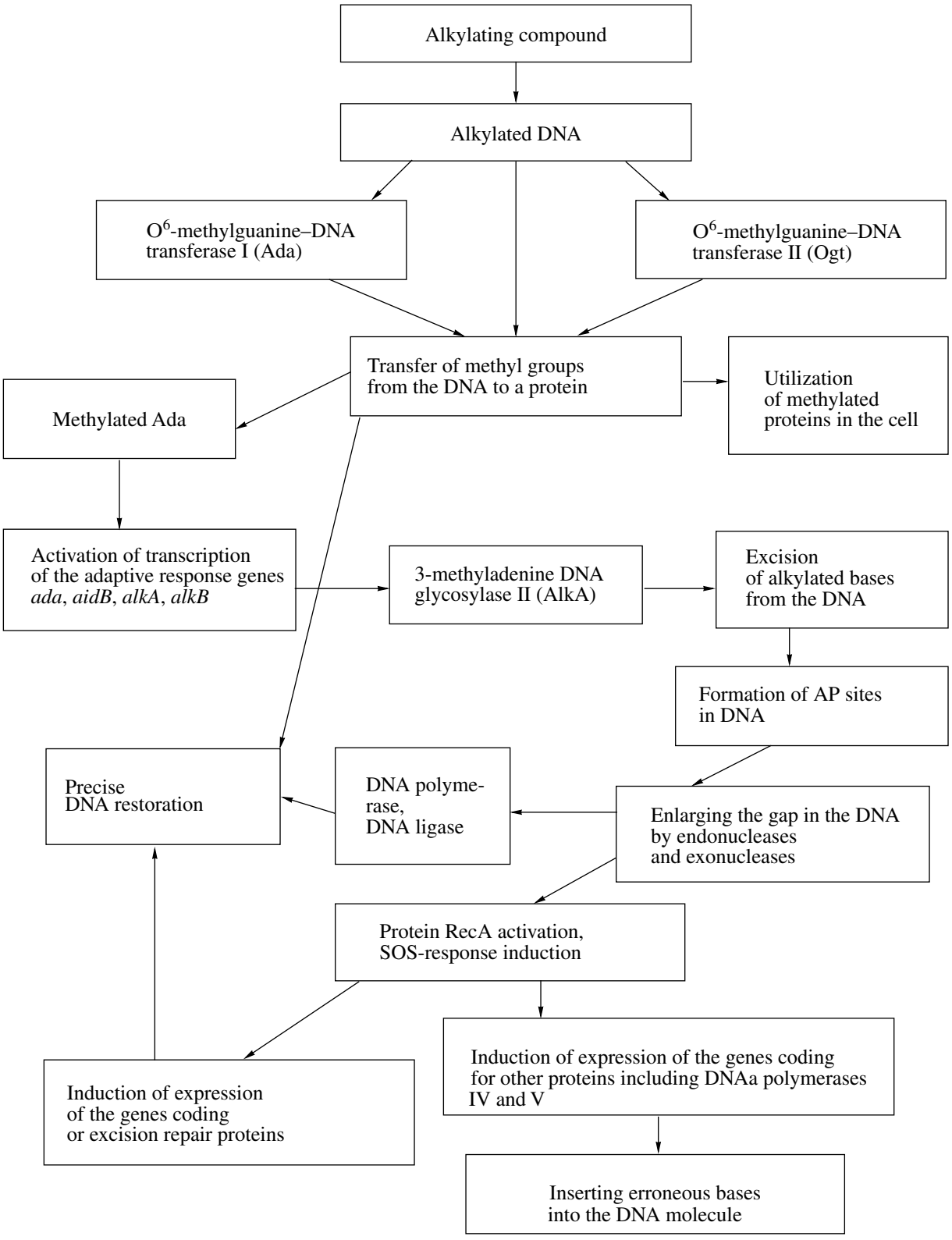

Fig. 1. Activation of various repair systems and the SOS response in *E. coli* cells following the effect of alkylating compounds.

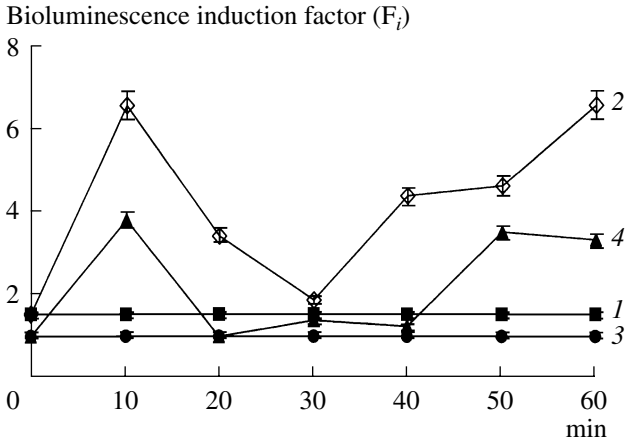

Fig. 2. Effect of osmotic stress and MNNG on SOS-dependent bioluminescence in the culture of *E. coli* strain WP2. The bioluminescence induction factor was determined in the cultures of cells not adapted (*1, 2*) and adapted (*3, 4*) to MNNG; *1* and *3*, optimal conditions; *2* and *4*, osmotic stress.

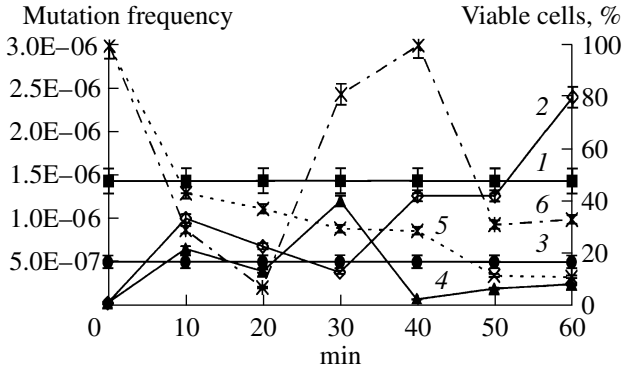

Fig. 3. Combined effect of osmotic stress and MNNG on *E. coli* WP2 cultures. Induced mutation frequency was determined in the cultures of MNNG-unadapted (*1, 2*) and adapted (*3, 4*) cells; *1* and *3*, optimal conditions; *2* and *4*, osmotic stress. *5* and *6*, cell viability in MNNG-unadapted and adapted cultures under osmotic stress.

[19]. It is well-established that the expression of the adaptive response inhibits the induction of some SOS genes. The SOS response diminishes the adaptive response [20]. Nevertheless, simultaneous activation of the SOS and adaptive responses in an *E. coli* cell is possible in the presence of protein RecF, although this protein is not implicated in any of these responses and its function is to stabilize the damaged DNA replication fork and to restore the disrupted replication process [21].

Our research focused on the question of whether the antagonistic relationship between the SOS response and adaptive DNA repair influences MNNG-induced mutation frequency. The induced mutation frequency and SOS response induction were monitored at 10-min intervals during the 1 h period of osmotic stress.

Research on the functional relationship between the bioluminescence induction factor (F_i) and osmotic stress duration revealed that nonadapted wild-type cells reached the maximum F_i values twice. The first peak value of the SOS-dependent bioluminescence operon induction occurred at the tenth minute of osmotic stress, and the second F_i increase was caused by a 60-min stress (Fig. 2). A comparison of the data on the dynamics of SOS-dependent bioluminescence with the mutation test results revealed that the highest MNNG-induced mutation frequency values in nonadapted wild-type cells also occurred at minutes 10 and 60 of osmotic stress (Fig. 3). The strongest bactericidal effect was detected in the subcultures that were incubated for 50 and 60 min under osmotic stress (Fig. 3).

The product of the *uvrA*$^+$ gene responsible for nucleotide excision repair is involved in recognizing the impaired DNA site and attaching the protein complex that secures its repair [12, 22]. The expression of the LexA-controlled p*cda*::*luxCDABE* transcription cross-link in the cells not adapted to MNNG, with mutant *uvrA* genes, peaked after 10 min of osmotic stress and remained unchanged after this point (Fig. 4). Our analysis of the induced mutation frequency in unadapted cells of this strain demonstrated that the cells' sensitivity to the mutagenic effect of MNNG gradually increased during the whole period of incubation under osmotic stress (Fig. 5). The strongest bactericidal effect was produced by MNNG in combination with a 10- and 60-min incubation under a high NaCl concentration (Fig. 5).

Studies with MNNG-adapted wild-type cells revealed that F_i peaked twice during the 1-h period of osmotic stress (Fig. 2), while only a single induced mutation frequency peak occurred at minute 30 (Fig. 3). Our analysis of the number of viable MNNG-

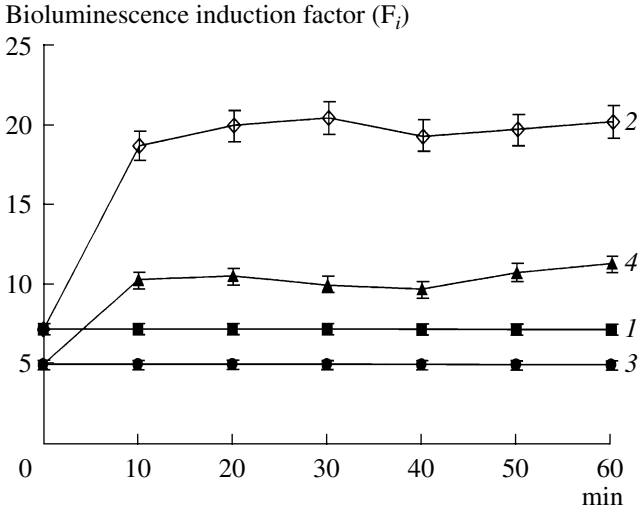

Fig. 4. Effect of osmotic stress and MNNG on SOS-dependent bioluminescence in the culture of the strain *E. coli* WP2s. See Fig. 2 for designations.

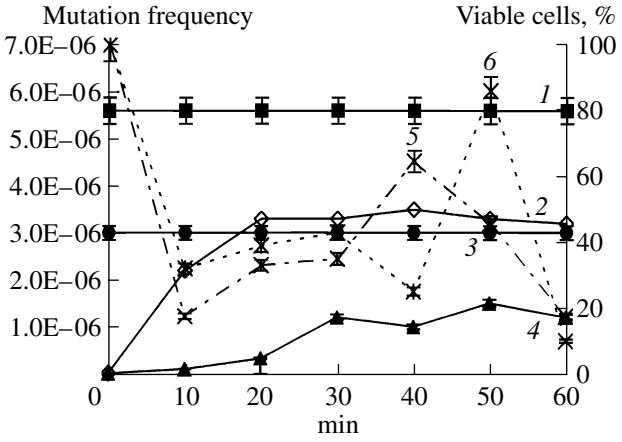

Fig. 5. Combined effect of osmotic stress and MNNG on *E. coli* WP2s cultures. See Fig. 3 for designations.

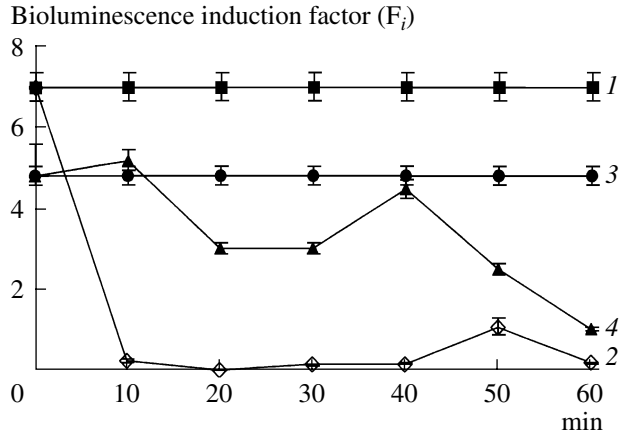

Fig. 6. Effect of osmotic stress and MNNG on SOS-dependent bioluminescence in the culture of the strain *E. coli* PJ2. See Fig. 2 for designations.

adapted wild type cells under osmotic stress demonstrates that induced mutagenesis activation and the bioluminescence induction factor are independent of cell death in the subcultures involved (Fig. 3).

The bioluminescence induction factor in MNNG-adapted, *uvrA*-mutant cells, and in unadapted cells, peaked after 10 min of osmotic stress and remained unchanged after this point. We obtained valid data that the F_i value in MNNG-unadapted cells is two times higher than in MNNG-adapted cells. Similar to the bioluminescence induction factor, the induced mutation frequency in adapted cells with mutant nucleotide excision repair genes reaches a maximum at minute 30 of osmotic stress, followed by a plateau (Fig. 4 and 5). Our analysis of viable cell numbers brought us to the conclusion that the bactericidal effect of MNNG and osmotic stress exert no influence on the induced mutagenesis in MNNG-adapted *E. coli uvrA* cultures (Fig. 5).

Thus, the first *cda* gene promoter expression peak in unadapted wild-type cells, which occurs after 10 min of incubation under osmotic stress, coincides with an increase in the induced mutation frequency. A subsequent decrease in the induced mutation frequency and F_i indicates the induction of the nucleotide excision repair mechanism in the cells. It is well-established that the SOS response is induced 22.5 min after the treatment of *E. coli* cells with DNA-damaging substances. The *uvrA*, *uvrB*, and *uvrD* genes that encode nucleotide excision repair proteins have weak SOS-dependent promoters that are among the first promoters to be activated [12]. The lack of induced mutation and the period of F_i decrease in the *uvrA*-mutant strain confirm this suggestion. Analogous results obtained in studies with MNNG-adapted cells of the above strains indicate that, despite the induction of the adaptive response, the combined effect of MNNG and osmotic stress results in SOS-response gene expression. The nucleotide excision repair mechanism turns on in the cells. The induction of the genes coding for nucleotide excision repair proteins during this period is consistent with the data available in the literature. Friedman et al. [23] reported that several peaks of expression of some SOS-dependent promoters were observed after damaging the DNA with UV radiation. After 20–25 min of irradiation with UV light (less than 10 J/m^2), a solitary *recA* and *lexA* gene promoter induction peak occurred [23], suggesting that a weak induction of these promoters is sufficient for activating the expression of nucleotide excision repair genes in the cells [24]. The temporal discrepancy between our results and the data reported in the literature may be due to the different mutagens involved or to the intricate regulation system of SOS-dependent promoters. It is known that SOS gene expression varies depending on the affinity of protein LexA for the promoter site and on the promoter structure [12], apart from the SOS box number and the variations in the palindrome structure.

Adaptive repair is the main mechanism of restoring alkylated DNA. Protein Ada plays the leading role in the adaptive response [1]. Protein RpoS, the σ^{38} subunit of the RNA polymerase, performs one of the key functions in protecting *E. coli* cells from osmotic stress and inducing the adaptive response [7]. Similar to the wild-type strain, *E. coli* cells of strain PJ2 not adapted to MNNG, which carry the *ada* gene mutation, demonstrate two F_i peaks at minutes 10 and 40 of osmotic stress (Fig. 6). The maximum F_i value occurred at minute 50 of osmotic stress in cells of this strain that were preincubated in a medium with a low MNNG concentration (Fig. 6). The induced mutation frequency in unadapted cells with the *ada* gene mutation peaked by minute 50 of osmotic stress. There were no significant differences in viable cell numbers among subcultures with various periods of incubation with NaCl (Fig. 7). Our analysis of the induced mutation frequencies and viable cell numbers in MNNG-adapted cells of strain PJ2 revealed that the highest induced mutation fre-

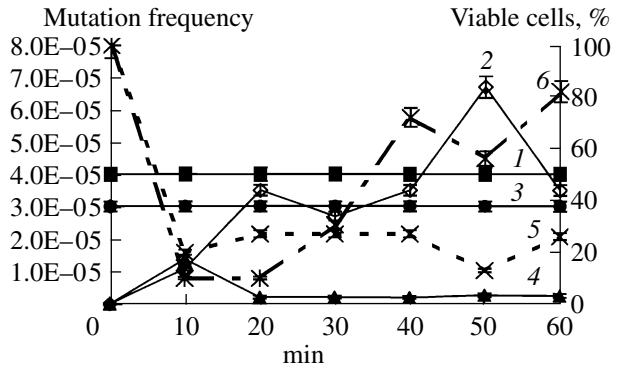

Fig. 7. Combined effect of osmotic stress and MNNG on *E. coli* PJ2 cultures. See Fig. 3 for designations.

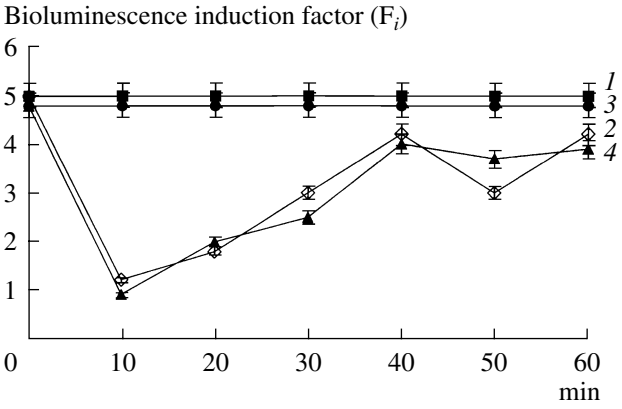

Fig. 8. Effect of osmotic stress and MNNG on SOS-dependent bioluminescence in the culture of the strain *E. coli* JC7623. See Fig. 2 for designations.

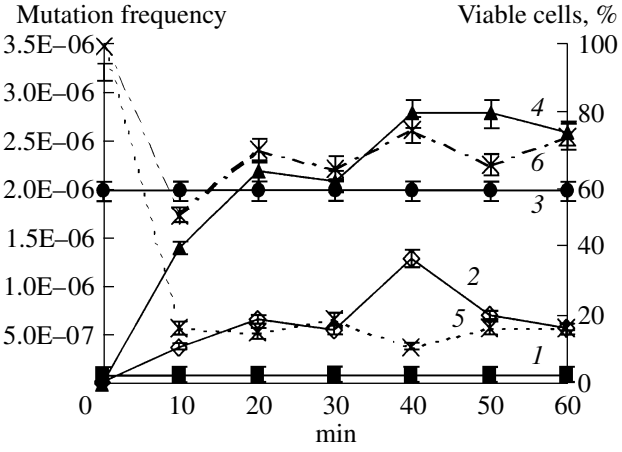

Fig. 9. Combined effect of osmotic stress and MNNG on *E. coli* JC7623 cultures. See Fig. 3 for designations.

quency value and the strongest bactericidal effect are attained at the initial stage of incubation with sodium chloride (Fig. 7).

The F_i values in the cultures of both MNNG-adapted and unadapted *E. coli* cells of strain JC7623 with a mutant *rpoS* gene peak after 40 min of osmotic stress (Fig. 8). These results are consistent with the mutation test data, i.e., the induced mutation frequency maximum occurs after 40 min of osmotic stress. The induced mutation frequency in MNNG-adapted cells is significantly higher than in unadapted cells (Fig. 9).

Presumably, the observed increase in induced mutation frequency during a 10-minute osmotic stress in the wild-type strain is due to the operation of DNA polymerase IV in the cell. A weak SOS-inducing signal is sufficient for inducing the *dinB* gene [25]. This suggestion is supported by the lack of the first peak in the cells of the strain with mutant *rpoS* genes, because *dinB* gene expression, apart from the SOS system, is controlled by protein RpoS [26].

A 40–50 minute osmotic stress results in increasing F_i and induced mutation frequency in wild-type *E. coli* and in the cells with mutant *ada* and *rpoS* genes. Probably, the transition from precise DNA restoration to SOS mutagenesis is made within this interval, involving proteins UmuDC (DNA polymerase V). The (UmuD')2-UmuC trimer displays DNA polymerase activity, and it nonspecifically incorporates deoxycytosine monophosphate (dCMP) in the growing DNA string. In this case, the number of deletions, frameshift mutations, and base-pair substitutions is increased [27]. Friedman et al. [23] reported that the first peak value of the *umuDC* operon promoter induction was attained 40 min after UV irradiation, which is consistent with our suggestion.

Hence, we obtained experimental data testifying to differences in the dynamics of F_i induction and induced reversion frequency during a 1-h osmotic stress in the cells of the wild-type strain and the strains with mutations in the *ada, rpoS*, and *uvrA* genes. Within the whole incubation period, these variables twice reached peak values in wild type *E. coli* cells. Only the second peak also occurred in *E. coli* cells with the mutant *rpoS* gene. No peaks were detected in *E. coli* cells with the mutant *uvrA* gene. The *uvrA E. coli* was characterized by an elevated expression of the SOS-dependent *lux* operon and an increased induced mutation frequency during the whole stress period, in contrast to the other strains examined. Based on the research results, we suggest that the *uvr* gene mutation accelerates the transition from precise DNA restoration to SOS mutagenesis under osmotic stress.

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