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EXPERIMENTAL ARTICLES

Effect of Stress on Emergence of Antibiotic-Tolerant *Escherichia coli* Cells

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Abstract—Effect of sublethal doses of physical and chemical stressors (heat shock for 2 h at 45°C and addition of C12-alkylhydroxybenzene, a microbial alarmone) on development of resistance to the subsequent lethal antibiotic attack and the role of the time interval between these treatments were studied on a submerged batch culture of *Escherichia coli* K12. The interval sufficient for the development of stress response provides for development of temporary adaptive resistance to the antibiotic attack, resulting in increased number of surviving persister cells. The interval below the time required for the stress response potentiates cell death and results in a decreased number of persisters. Heterogeneity of the fractions (10^{-4} to 10^{-2}% of the initial CFU number) surviving lethal doses of an antibiotic (ampicillin or ciprofloxacin) was found. Apart from a low number of antibiotic-resistant cells (up to 0.005% of surviving cells), the fractions contained antibiotic-tolerant forms, such as temporarily resistant metabolically adapted cells, long-term surviving persisters, and the cells of slowly growing SCV variants with small colonies ($d \leq 1$ mm). Persisters are hypothesized to act as precursors for cystlike dormant cells (CLC), in which the cell differentiation stage is completed and the processes of cell ametabolism (transition to the anabiotic state) are still incomplete.

Keywords: *Escherichia coli* K12, stress, alkylresorcinol, antibiotic-tolerant cells, persisters, adaptively resistant cells, SCV variants, persisters as precursors of cystlike dormant forms

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Elucidation of the basic principles of drug tolerance of pathogenic and opportunistic bacteria is becoming increasingly important, given the decreasing efficiency of available antibiotics (Gillings and Stokes, 2012; Millar, 2012). A large number of long-term survivors (persister) cells were detected in permafrost microbial associations that are hundreds of thousands to millions of years old, and the genetic determinants of antibiotic tolerance and the pathways of their horizontal transfer were investigated. The conclusion was drawn that the system of adaptive tolerance to antibiotics took shape long before the onset of the anthropogenic impact on the environment (Petrova et al., 2012; Shapiro and Dworkin, 1997; van der Woude, 2011).

A prerequisite for fruitful research on the causation of high resistance of microorganisms to damaging factors, including antibiotics, is interpretation of microbial populations as peculiar self-regulating multicellular organisms (Shapiro and Dworkin, 1997). Their main feature is the phenotypic and genotypic heterogeneity of the population, which provides for the survival of the organism population under the stress caused by a drug or any other factor (van der Woude, 2011).

The main strategies of population survival in the presence of an antibiotic are to be considered in the light of these statements. The first of them is based on availability and expressibility of the genes responsible for the changes in the targets for antibiotics, efficient extrusion of antibiotics from the cells via efflux systems, their modification or destruction, etc. (Guilfoile and Alcamo, 2007). Such inheritable antibiotic resistance genotypes enable the cells to actively resist the impact of a drug preparation and to divide in its presence (Guilfoile and Alcamo, 2007; Grassi and Grilli, 2012; Petrova et al., 2011). Inoculation onto a fresh medium results in the growth of the same genotypically antibiotic-resistant cell populations.

The second evolutionarily developed strategy of bacterial survival in the presence of bactericidal antibiotic doses involve cell differentiation processes that result in altering the phenotype, decelerating cell growth, disrupting the cell cycle, and inactivating antibiotic targets (Gefen and Balaban, 2008; Lewis, 2010; Kint et al., 2012; Balaban et al., 2013). Using this

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uninheritable passive mechanism of antibiotic tolerance, the cells remain viable in the presence of antibiotics, although they do not divide. Upon transfer onto a fresh medium, such cells give rise to a population that is similar to the parental population, with the majority of its cells sensitive to the antibiotics, although a small number of antibiotic-tolerant cells forms again (Gefen and Balaban, 2008; Lewis, 2010; Kint et al., 2012; Balaban et al., 2013). Such antibiotic-tolerant forms were denoted as persisters by Bigger (1944).

Currently, two types of persisters are distinguished: type II persisters (PII), which develop in a growing culture during the exponential and the growth deceleration stage and account for about 0.01% of the population, and type I persisters (PI), which appear in a stationary-phase culture; their percentage amounts to 1% and above (Balaban et al., 2004; Shan et al., 2006).

Another tolerant phenotype is characteristic of the so-called indifferent cells, whose physiological state is typical of the stationary-phase culture cells (Levin, Rozen, 2006). Disrupting the cell cycle provides for their temporary capacity to survive in the presence of antibiotics. Unlike persisters, they are sensitive to quinolone antibiotics, including ciprofloxacin (Grant and Hung, 2013). Since both indifferent cells and type I persisters occur during the stationary phase, distinguishing between these two kinds of tolerant cells is of considerable importance. Another form of temporary bacterial tolerance to antibiotics is adaptive metabolic resistance that implicates changes in the permeability of cell envelopes (Fernandez and Hancock, 2012). Antibiotic-tolerant forms also include the SCV phenotype cells that form very small colonies ($d \leq 1$ mm) which grow extremely slowly and are therefore resistant to antibiotic agents (Grant and Hung, 2013). Among all antibiotic-tolerant forms, special attention is presently given to persisters because they survive in the presence of all antibiotics (antimicrobial preparations) and occur, as a surviving phenotype, in populations of antibiotic-resistant clones. Presumably, they are responsible for relapses of chronic infections. Importantly, persisters always form in a developing culture, irrespective of the presence of antibiotics that only represent the factors which select for persisters, in contrast to the pool of antibiotic-sensitive cells that are lysed.

There is evidence that unfavorable growth conditions such as nitrogen limitation (starvation) (Nguyen et al., 2011), hypoxia, and low pH values (Wayne and Lin, 1982) positively influence the formation of tolerant cells, i.e., transition from the conventional to the tolerant phenotype. Virtually no data are available on the relationship between the stress response of a population and the development of various antibiotic tolerance forms. The subsequent fate of persister cells under growth arrest conditions (on antibiotic-containing media) remains uncertain.

The goal of the present work was to investigate the impact of stress caused by heat shock or by addition of a microbial alarmone to the culture on the development of antibiotic tolerance of *E. coli* K12 cells and its type, to determine the heterogeneity of the subpopulation that survives after the addition of the antibiotic, and to determine the characteristics of the cells that survived after long-term (30 days) treatment with an antibiotic.

MATERIALS AND METHODS

The *subject of this work* was the gram-negative bacterium *Escherichia coli* K12 from the collection of the Gause Research Institute of New Antibiotics of the Russian Academy of Medical Sciences.

Cultivation. The bacteria were grown in the Luria–Bertani medium (LB Broth, Miller) with the following composition (g/L): yeast extract, 10.0; peptone, 5.0; NaCl 5; pH 7.0. The inoculum was an early stationary phase (20 h) culture added at a concentration that yielded the initial experimental culture density of 8.0×10^7 cells/mL. Aliquots of the experimental culture (1 mL) were transferred into 2-mL Eppendorf test tubes. The culture was grown with stirring on a BIOSAN TS-100 thermoshaker (700 rpm) at 28°C.

Detection of antibiotic-tolerant cells (type II persisters) was performed using the modified Keren method (Keren et al., 2004). The *E. coli* K12 cells of the experimental and the control variant were grown as described above until they reached the exponential growth phase. Thereupon, the experimental test tubes were supplemented with the antibiotics ampicillin (Amp) or ciprofloxacin (Cip) at concentrations of 30 or 100 μg/mL and further incubated on a thermoshaker under the same conditions. An equivalent amount of distilled water was added to the control culture. To determine the number of viable cells in the experimental and control (antibiotic-free) variants, the necessary number of samples were placed in Eppendorf tubes; the cells were precipitated by centrifugation (10000 *g*, 5 min) and washed twice with normal saline (0.9% NaCl). The cell pellet was resuspended in 1 mL of normal saline and used for determining the CFU number on agar-containing LB medium with or without Amp (10 μg/mL).

Detection of antibiotic-tolerant cells forming under the influence of heat shock. *E. coli* K12 cells were grown as described above until they reached the exponential growth phase (CFU = 5.3×10^8 cells/mL). The test tubes of the control variants were supplemented with Amp at concentrations of 0, 10, 30, 60, and 100 μg/mL, and the cultivation on a thermoshaker (700 rpm; 28°C) was continued. The experimental variants were incubated at a sublethal temperature (45°C as determined in preliminary studies) using a thermoshaker (700 rpm), whereupon the antibiotic Amp was added to the test tubes at concentrations of 0, 10, 30, 60, and 100 μg/mL. The test tubes with both

variants were further incubated on a thermoshaker (700 rpm, 28°) for 7 days and, subsequently, for 30 days under static conditions. Samples were taken at regular intervals in order to determine the titer of viable cells.

Influence of a microbial alarmone, C12-alkylhydroxybenzene (C12-AHB), on the formation of antibiotic-tolerant cells. *E. coli* K12 cells were grown as described above until they reached the exponential growth phase (CFU = 5.3×10^8 cells/mL). Thereupon, C12-AHB solution (Sigma, United States) was added to the experimental variants at a concentration of 2.0×10^{-4} M (the volume added was 10 μL). After 30 min, Amp (10 μg/mL) or Cip (100 μg/mL) was added. Instead of C12-AHB, the control cultures were supplemented with 10 μL of the solvent (ethanol). The test tubes containing the control and experimental variants were incubated on a thermoshaker (700 rpm, 28°C) for 2 to 7 days. Samples were taken at regular intervals in order to determine the titer of viable cells.

Cell viability was determined from the colony-forming unit number (CFU/mL) using a modified Koch method. Samples (100 μL) were taken aseptically from the cell suspensions of the control and experimental variants, and a number of tenfold dilution were prepared. Aliquots (5 μL) from these dilutions were inoculated onto the surface of agar-containing medium (1.5 % agar) to produce point colonies on Petri dishes (10 points per dilution). To determine the total number of surviving cells in the presence of an antibiotic, LB and LB/2 media were used for inoculation. The number of antibiotic-resistant clones was determined using the medium with Amp, which was added to the agar medium at a concentration of 10 μg/mL. Upon inoculation, the plates were incubated at 28°C. The resulting colonies were counted on day 3, 5, 7, and 10 of incubation, and their colony morphology was noted. The CFU/mL number was determined as the mean value of the results of three repeats with ten inoculations for each dilution. Three repeats of each experiment were conducted. The diagrams and tables contain the results of typical experiments.

Microscopic studies were conducted using a Reichert microscope (Austria) with a phase contrast device. Live and dead cells were distinguished by staining them with the Live/Dead dye (Baclight kit L13152, Molecular Probes). For electron microscopic studies, the cells were collected by centrifugation and fixed by a standard method. Ultrathin sections were contrasted with 3% uranyl acetate solution in 70% ethyl alcohol for 3 min and stained with lead solution for 5 min according to Reynolds. The sections were examined in a ZEM-100B electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

The phenotypic variability of the bacterial populations was determined from the development, on agar-containing media, of colony morphology variants that differed from the dominant variant in terms of colony size, form, consistence, and color. The variation index of bacterial populations was determined by plating on LB agar (or LB agar with 10 μg/mL Amp) as a certain percentage of colonies of a specific colony morphology type of the total number of growing colonies.

Statistical analysis was carried out by standard mathematical methods, using the Student *t*-test and calculating the mean square deviation, with Microsoft Excel XP software. A data array was considered homogeneous if the mean square deviations (σ) did not exceed 10%. The differences between data arrays were considered significant if the probability criterion (*P*) exceeded 0.95.

RESULTS

Detecting Antibiotic-Tolerant Type II Persister Cells in the Batch Culture of E. coli K12

It was established earlier (Keren et al., 2004; Zhang, 2014) that some of the cells of an initially antibiotic-sensitive population disrupt their cell cycle in submerged batch culture. As a result of cell differentiation, they acquire a tolerant phenotype that enables them to survive in the presence of antibiotics. The frequency of such persister cells in a developing culture varies depending on its age (Lewis, 2010; Kint et al., 2012; Balaban et al., 2013). These data were confirmed in our studies with a submerged culture of *E. coli* K12 (Fig. 1). The share of cells that remained viable after antibiotic treatment increased with an increase in the culture age (Fig. 1a). It also depended on the kind and dose of the antibiotic. It was lower with ampicillin than with ciprofloxacin (Fig. 1b). It also decreased during the incubation with the antibiotic (during the first 7 days of incubation) (Figs. 1a and 1b). For instance, the percentage of surviving cells was 0.02% 4 h after the addition of Amp (100 μg/mL) to a 2-h growing culture. After 7 days, approximately 10 cells per 1 mL retained the capacity to form CFUs. In the presence of Cip (100 μg/mL), the percentage of survivors was 0.2% after 4 h, and it decreased to 0.0006% after 7 days of incubation.

Importantly, the subpopulation obtained by antibiotic treatment was heterogeneous in terms of the mechanisms enabling the cells to survive. It contained type II antibiotic-tolerant persister cells and a small number (up to 0.006% of survivors in 3-h samples) of antibiotic-resistant cells that were detected by plating the samples on LB medium with ampicillin (Table 1). Therefore, this antibiotic-resistant subpopulation will be considered a component of the persister fraction in the subsequent sections of this work. Moreover, development of the cultures on the Amp-containing medium resulted in a substantial change in the spectrum of surviving subpopulation, which favored the segregation and development of minor colony morphotypes.

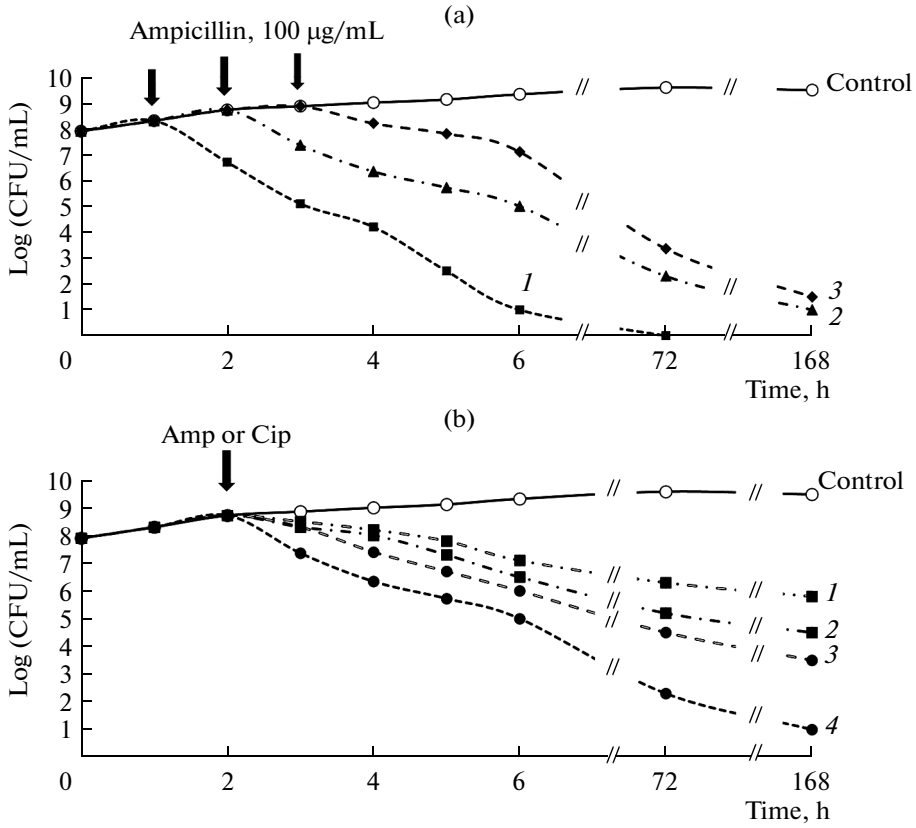


Fig. 1. Cell death curves of *E. coli* K12 cultures in the presence of antibiotics: a: ampicillin (100 μg/mL) added to the culture after 1 h (*1*), 2 h (*2*), and 3 h (*3*) of growth; b: ciprofloxacin at concentrations of 30 (*1*) and 100 (*3*) μg/mL and ampicillin at concentrations of 30 (*2*) and 100 (*4*) μg/mL.

Effect of Stress on the Formation of Antibiotic-Tolerant Cells in E. coli K12 cultures

It was revealed in a number of works that the number of antibiotic-tolerant persister cells is larger in nitrogen-starved cultures than in those without the limitation (Grant and Hung, 2013). No data are available concerning the role of short-term stress caused by environmental changes in terms of formation of antibiotic-tolerant cells. In this study, stress factors influencing the development of an *E. coli* K12 culture were exemplified by high (supraoptimal) temperature, including its sublethal dose (which was established in preliminary studies) and alkylhydroxybenzenes (alkylresorcinols), chemical analogs of microbial regulatory substances which function as alarmone (alarm signal).

Effect of heat shock on the formation of antibiotic-tolerant cells. In the experimental systems, an *E. coli* K12 population was grown in Eppendorf tubes until it reached the exponential stage (CFU = 5.3×10^8 cells/mL), and was then exposed to a sublethal effect of high temperature (45°C, 2 h). Under these conditions, bacterial cells stopped growing and dividing but did not lyse. This was established by phase contrast microscopy and also by determining the number of viable cells (CFU/mL) during heat treatment. After 2 h, the heat shock-exposed cultures were treated with the antibiotic ampicillin at concentrations of 10, 30, 60, and 100 μg/mL in order to determine the fraction of survivors (persister cells) (Fig. 2, grey dashed lines). In the control systems, the antibiotic was added to the populations that have not been exposed to heat shock (Fig. 2, black dashed lines). Since stressed cells stopped dividing, their numbers upon addition of the antibiotic were identical in the experimental and control samples. Analysis of the results revealed that, 2 h after Amp addition (4 h of incubation in the control samples and 6 h of incubation in the experimental samples), the number of survivors in the stressed populations was 2–3 orders of magnitude higher than in the control populations (Table 2, Fig. 2). It was also revealed that the survivor number depended on the antibiotic dose in the control systems, while no manifest dependence of the survivor number on the antibiotic dose was established in the experimental systems. However, the pattern changed after 72 h of incubation. The dose dependence became more pronounced in the experimental systems where the difference between survivor numbers at different antibiotic concentrations (within the 10–100 μg/mL concentration range) was four orders of magnitude (Fig. 3a). In the

Table 1. Detection of antibiotic-resistant strains in an *E. coli* K12 population and the persister subpopulation

Culture age, OD	Colony number (CFU/mL) on the medium with Amp (μg/mL)	
	0	10
3 h, OD 1.0	2.3×10^8 (100)	1.3×10^4 (0.006*)
5.5 h, OD 2.5	7.9×10^8 (100)	4.2×10^4 (0.005)
7 h, OD 4.1	1.9×10^9 (100)	3.5×10^5 (0.02)
Persisters (Amp, 100 μg/mL)**		
3 h***	2.7×10^7 (100)	1.6×10^3 (0.006)
24 h***	4.7×10^2	0

* In parentheses, % of the colonies of each variant that grow on the medium with the antibiotic.

** Persisters were obtained by adding Amp (100 μg/mL) to an exponentially growing 2 h culture.

*** Persister age: 3 and 24 h after adding Amp.

variants with an Amp dose of 10 μg/mL, the populations resumed their growth (the samples were taken after 168 h of incubation), and their cell number increased almost to the level in the control systems (without Amp). After 168 h of incubation (Fig. 3b), the survivor number drastically dropped in both the experimental and the control systems. However, it was significantly higher (by 1.5–2 orders of magnitude) in the experimental, heat shock-pretreated, systems. The maximum number of survivors (persister cells) was revealed in the experimental systems with an Amp dose of 30 μg/mL. At this Amp dose, the difference between the control and experimental systems was also the highest (Fig. 3b).

Hence, one of the effects of stress caused by exposing bacterial populations to heat shock two hours before adding an antibiotic was the development of capacity for short-term survival (for 2 h) in the presence of the antibiotic. This phenomenon may be accounted for by development of adaptive resistance in the overwhelming majority of the cells under the influence of heat shock, a factor that induces a stress response, including changes in cell permeability, which may impede the influx of the antibiotic into the cell (Nguyen et al., 2011). In this state, most of the

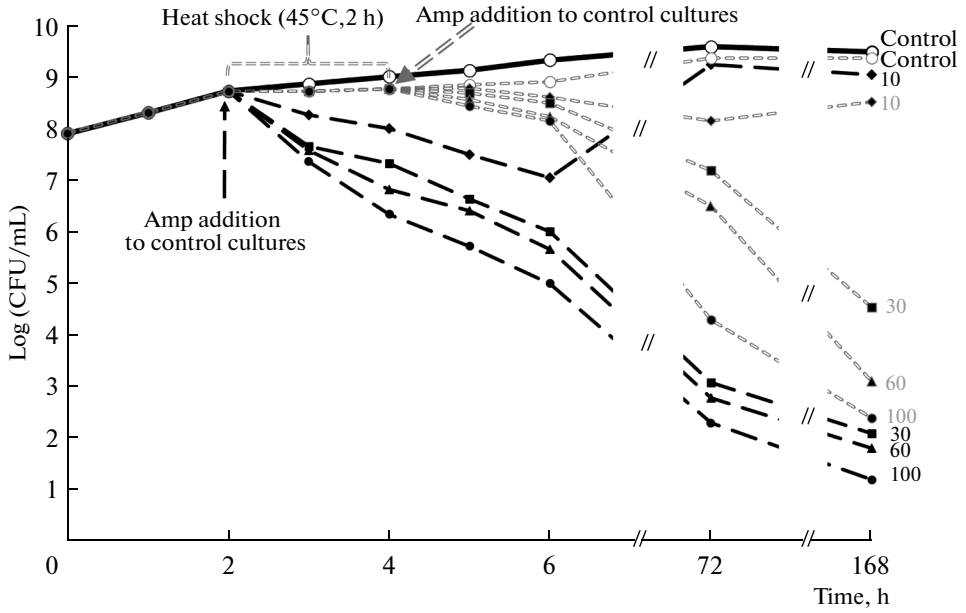


Fig. 2. Viability of *E. coli* K12 cells (CFU/mL) after adding Amp (10, 30, 60, and 100 μg/mL) compared to control cultures (solid lines) and heat shock-pretreated cultures (45°C, 2 h) (grey dashed lines). Numbers at the curves, the antibiotic (Amp) dose, μg/mL.

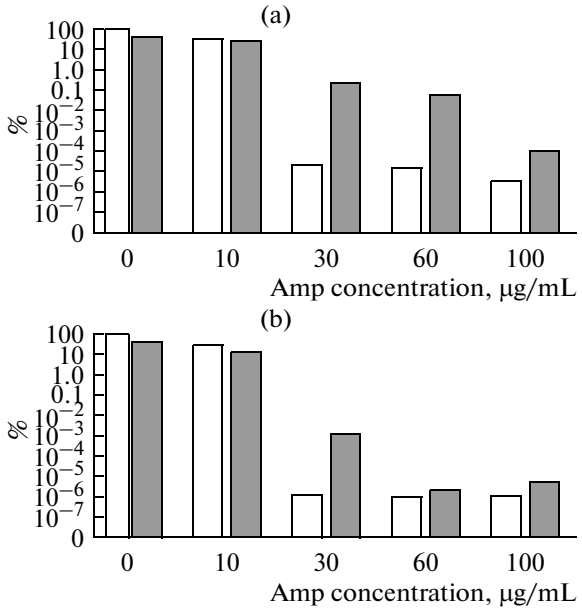


Fig. 3. The number of cells (%) that remained viable for 3 (a) and 7 (b) days after the addition of Amp to heat shock-pretreated (dark columns) and control (white columns) cells of *E. coli* K12. 100%, initial CFU/mL value in the culture of the control variant (without heat shock and the antibiotic).

cells in a population temporarily cease dividing and, during the division arrest period, retain their viability in the presence of the antibiotic. After 2 h of incubation at 28°C, stressed cells start to restore their metabolic activities. Therefore, the cells in the experimental systems that have not been treated with the antibiotic resumed their growth and division. In contrast, metabolically adapted cells started perishing in the antibiotic-treated experimental systems (Fig. 2). The true persister cells resulting from specific cell differentiation remained resistant not only to the short-term antibiotic attack, but also to long-term incubation in an antibiotic-containing medium, i.e., under prolonged growth arrest conditions (Figs. 2 and 3). It should be noted that both adaptively tolerant cells and true persisters should be classified as tolerant cells according to the distinctive criterion (viability retention in the presence of the antibiotic).

Another effect of stress caused by heat shock (2 h, 45°C) was an increase in the number of long-term survivors of persister cells (retaining viability for seven and more days) in comparison to the unstressed control cultures. This was particularly manifest in the variants with 30 and 60 μg/mL ampicillin (Fig. 2). The differences in the sensitivity of the clones to increasing Amp doses both in the control (unstressed) and experimental (exposed to heat shock) variants indicated the populations' physiological heterogeneity in this respect. In a similar fashion, the results of comparing the CFU numbers after inoculation on the standard and diluted medium provided evidence for the physiological heterogeneity of the persister fraction in the control and experimental variants. The heat shock-preadapted subpopulation of temporarily tolerant, metabolically active cells was found to be more homogeneous. After addition of the antibiotic, the difference in CFU number was less significant in the experimental variants than in the control ones (unstressed cells). After 72 h, this difference disappeared (Table 3). Of special interest was a decrease in the physiological heterogeneity level in terms of colony morphology characteristics in heat shock-stressed population (see section 3).

Hence, the development of the state of stress in the cells preadapted them to the subsequent antibiotic treatment and significantly influenced the antibiotic tolerance of the bacterial population, providing for short-term adaptive tolerance of all its cells and enhancing the intensity of persister formation.

Influence of the regulatory metabolites with alarmone activity on formation of antibiotic-tolerant persister cells. In the next series of experiments, the time interval between the stress action and the subsequent addition of the antibiotic was decreased to 30 min so that the stress response did not develop. C12-AHB, a chemical analog of bacterial density-dependent regulators alkylhydroxybenzenes, AHB (El'-Registan et al., 2006), was used as a stressor. At the population level, AHB act as alarmones (alarm signals), i.e., extracellular simulacra of stressors (El'-Registan et al., 2006). At the cellular level, they activate the expression of the stress genes involved in the SOS response and the RpoS regulon (Golod et al., 2009), decrease membrane microviscosity (El'-Registan et al., 2006), and modify the tertiary structure of proteins (Krupyanskiy et al., 2012). Addition of C12-AHB (2×10^{-4} M) to an *E. coli* K12 culture during the exponential growth stage temporarily (for 4 h) inhibited its growth; thereupon, growth resumed and reached the control level, indicative of the development of the stress response. In the experimental systems, the antimicrobial effect of the antibiotic was conspicuously enhanced if Amp (10 μg/mL) was added to the stressed cultures 30 min after adding C12-AHB. This resulted in the development of distress and cell death (Table 4). In the control systems (without C12-AHB), ampicillin at the sublethal concentration exerted a very weak bactericidal influence during the whole observation period (48 h of growth). The same effect occurred if another antibiotic, Cip, was applied at a bactericidal concentration of 100 μg/mL. The survivor number with Cip alone was 3–4 orders of magnitude higher than after the combined effect of Cip and C12-AHB (Table 4). Importantly, the number of survivors capable of CFU formation was crucially lower in the 2-day experimental systems than in the respective control systems: it decreased to 10 cells/mL in the experimental variants with 10 μg/mL Amp.

Table 2. Changes in cell number (CFU/mL) and the phase variant spectrum (share, %) of the survivor fraction obtained after the addition of the antibiotic Amp to heat shock-pretreated *E. coli* K12 cultures and to the control cultures (without pre-adaptation)

Amp concentration, μg/mL*	Sampling time, h**	Without pre-adaptation							Heat shock-pretreated						
		CFU/mL	Phase variant percentage						CFU/mL	Phase variant percentage					
			S	Sl	Pl	Sm	SCV	Total***		S	Sl	Pl	Sm	SCV	Total***
0	2	8.0×10^8	100	0	0	0	0	0	8.0×10^8 (100****)	100	0	0	0	0	0
	72	3.8×10^9	100	0	0	0	0	0	2.3×10^9 (60.5)	100	0	0	0	0	0
	168	3.0×10^9	89.3	0	0	6.7	4.0	10.7	2.3×10^9 (76.7)	95.7	0	0	1.4	2.9	4.3
10	2	1.0×10^8	100	0	0	0	0	0	4.0×10^8 (400)	100	0	0	0	0	0
	72	1.7×10^9	100	0	0	0	0	0	1.4×10^8 (8.2)	100	0	0	0	0	0
	168	1.1×10^9	94.5	0	0	2.2	3.3	5.5	3.2×10^8 (29.1)	90.7	0	0	0	9.3	9.3
30	2	2.1×10^7	94.6	0	0	2.4	0	5.4	3.1×10^8 (1.5×10^3)	100	0	0	0	0	0
	72	1.2×10^3	64.0	15.8	0	8.2	12.0	36.0	1.5×10^7 (1.3×10^6)	90.1	3.1	0	3.3	3.5	9.9
	168	1.3×10^2	45.5	0	0	15.0	39.5	54.5	3.5×10^4 (2.7×10^4)	94.2	0	0	1.5	4.3	5.8
60	2	6.6×10^6	85.0	3.2	0	2.8	9.0	15.0	1.7×10^8 (2.6×10^3)	100	0	0	0	0	0
	72	6.0×10^2	41.8	41.6	0	8.3	8.3	58.2	3.1×10^6 (5.2×10^5)	72.3	5.2	0	4.6	17.9	27.7
	168	6.5×10^1	28.3	0	0	56.3	15.4	71.7	1.3×10^3 (2.0×10^3)	60.4	0	0	39.6	20.0	39.6
100	2	2.2×10^6	72.8	3.0	0	15.1	9.1	27.2	1.4×10^8 (6.4×10^4)	100	0	0	0	0	0
	72	2.0×10^2	26.3	24.1	0	24.2	25.4	73.7	2.0×10^4 (1.0×10^4)	71.5	7.1	0	7.1	14.3	28.5
	168	1.6×10^1	0	0	0	67.7	33.3	100	2.5×10^2 (1.6×10^3)	21.2	0	0	58.3	20.5	78.8

* Antibiotic (Amp) was added to the *E. coli* K12 culture at time points given in Fig. 2.

** Culture incubation time after the addition of the antibiotic.

*** Total percentage of minor variants.

**** In parentheses, % of the CFU number of the same variant without pre-adaptation.

Table 3. Comparison of the cell numbers (%) in the control and heat shock-pretreated populations plated on the standard (LB) and diluted (LB/2) medium after adding Amp

Variants (Amp concentration, μg/mL)	Cell numbers (CFU/mL) obtained on the diluted medium (LB/2) expressed as % of the cell number obtained on the standard medium (LB)	
	Control population	Adapted cells
4 h after the onset of the experiment		
0	81.0	93.8
10	30.9	90.2
30	58.0	88.4
60	67.4	76.5
100	96	85.7
72 h after the onset of the experiment		
0	100	82.6
10	70.6	90.6
30	83.3	85.4
60	100.0	106.7
100	121.2	100

Table 4. Numbers of type II persisters (CFU/mL) obtained after addition of the antibiotics Amp (10 μg/mL) or Cip (100 μg/mL) to (i) *E. coli* K12 cultures preadapted with C12-AHB (2×10^{-4} M) or (ii) control cultures (without preadaptation)

Time of incubation with antibiotic, h		Control cultures (without pre-adaptation)	Cultures preadapted with C12-AHB*, 2×10^{-4} M
Amp, 10 μg/mL	3	2.5×10^8	1.1×10^3
	24	8.0×10^7	32
	48	2.5×10^8	10
Cip, 100 μg/mL	3	2.5×10^7	5.0×10^3
	48	6.3×10^5	4.7×10^2
	168	3.1×10^3	0

*An exponentially growing *E. coli* K12 culture was supplemented with C12-AHB (2×10^{-4} M) and thereafter, 30 min later, with antibiotics (Amp or Cip).

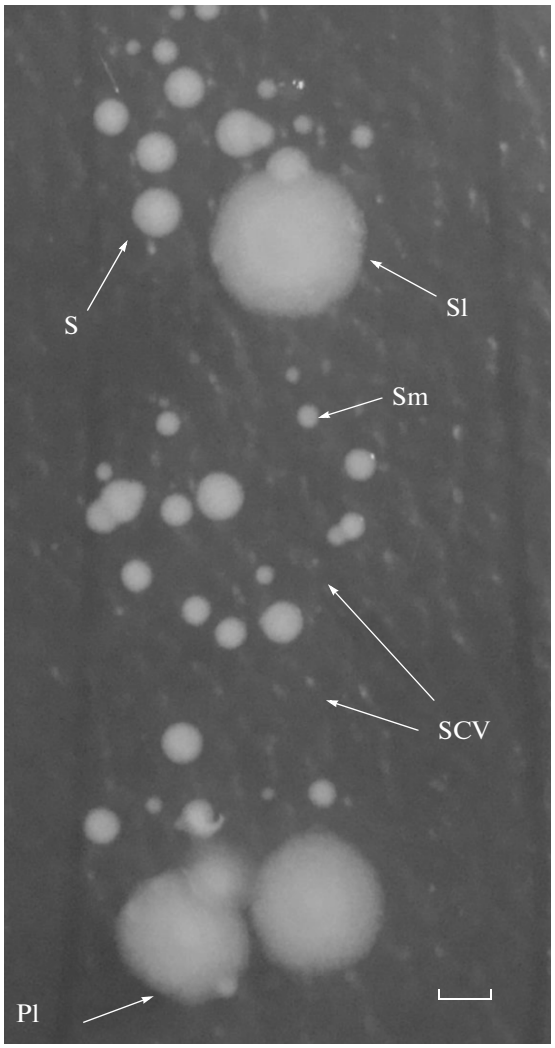
Hence, development of the mechanisms of antibiotic tolerance was strongly influenced by the stress state of the cells, which preadapted them to the subsequent effect of the antibiotic or, conversely, resulted in distress. The outcome varied depending on the time interval between the stress and addition of the drug preparation.

Heterogeneity of Antibiotic-Tolerant Survivor Population in Terms of Colony Morphology

The heterogeneity of the survivor population with respect to a physiological feature, the sensitivity to an antibiotic, was accompanied by the colony morphology heterogeneity of the survivor population. This was revealed by detecting antibiotic-resistant clones or antibiotic-tolerant persisters. The persister fraction of *E. coli* K12 was heterogeneous with respect to the morphotypes of the colonies that grew after plating on fresh media (Table 5). The population that grew on solid media contained, apart from the colonies of the dominant S type ($d = 4–6$ mm), the cells of the minor variants Sl and Pl with large colonies ($d = 10–16$ mm) and Sm with small colonies ($d = 2–3$ mm), whose percentage and number increased with an increase in the antibiotic dose and incubation time. There were also cells that produced SCV-type microcolonies with a diameter $d < 1.0$ mm (Tables 2 and 5). The latter type of colonies receives much attention currently, because they implement a strategy of extracellular bacterial persistence (Proctor et al., 2006).

Interestingly, the subpopulations of adaptively tolerant cells were more stable in terms of their phase variation spectrum after medium-to-long incubation

Table 5. Description of the colonies of the phenotypic variants of *E. coli* K12 obtained on the standard LB medium (1.5% agar) with or without Amp. Scale bar, 5 mm

Type	Description	Diameter, mm	Appearance
S	Rounded smooth convex greyish-light blue colonies	4–6	
Sl	Rounded smooth colonies with blurred edges, several times larger than S colonies	12–16	
Pl	Rounded smooth semitransparent colonies with ragged edges, several times larger than S colonies	10–16	
Sm	S-type colonies, the size being 1.5–2 times smaller than that of conventional S colonies	2–3	
SCV	Very small, almost transparent colonies	0.5–1.0	

periods. The percentage of minor phenotypes was 30 to 75% in the control unstressed systems 1–3 days after addition of the antibiotic (Amp, 30–100 μg/mL), whereas it was only 10 to 30% in the experimental, heat shock-exposed systems, and they developed by day 3 or later (Table 2).

Importantly, SCV-type microcolonies were only detected after a long cultivation time (after day 3) in dilutions with low cell concentrations or on the medium with the antibiotic (LB + Amp). Under standard conditions, the growth of this slowly growing and, therefore, antibiotic-tolerant phenotype was suppressed by the rapidly growing colonies of other morphotypes.

Hence, the preadaptation (heat shock) to the effect of an antibiotic did not only secure the temporary survival of the cells in the presence of an antibiotic, which was due to the decrease in cell metabolism and the development of the stress response. It also provided for long-term retention of the dominant morphotype, which is distinguished by its colony morphology characteristics.

Microscopic Examination of Survivor Subpopulations and Ultramicroscopic Characterization of Surviving Cells

Microscopic analysis of the subpopulation surviving Amp treatment of *E. coli* K12 in the control and experimental (after the heat shock) systems revealed its morphological heterogeneity in terms of cell size and the formation of refractile cells in 7–30 day cultures that survived after the addition of high ampicillin doses (60–100 μg/mL). Intense light refraction is peculiar to dormant anabiotic forms of microorgan-

isms such as spores, cysts, and cyst-like dormant cells (CLC) of bacteria (El'-Registan et al., 2006). Therefore, the increase in refractility in persister cells during long-term incubation probably indicated the maturation of persister cells and their conversion into dormant forms such as CLC. This conclusion did not contradict the results of our studies on the ultrastructural organization of survivors in the experimental and control systems after one month of incubation (Fig. 4–6). The cells displayed the visual features of the dormant forms of gram-negative bacteria that were earlier detected in laboratory cultures of bacteria stored for a long time (for 1 to 36 months) and grown both in liquid and on solid media (Bukharin et al., 2005), which have been reported for *Pseudomonas aurantiaca* (Mulyukin et al, 2008b) and *Azospirillum brasilense* (Mulyukin et al., 2009b), as well as in the dormant microbial associations of permafrost subsoil deposits of the Siberian tundra (Soina et al., 2004; Kryazhevskikh et al., 2013). *E. coli* K12 cells surviving for a long period of time (1 month) formed during the developmental cycle of bacterial cultures. They were characterized by resistance to stressors (antibiotics), peculiar ultrastructural features that testified to major intracellular alterations (not limited to age-dependent changes), and retention of the colony-forming capacity. Unlike the CLCs that formed in post-stationary bacterial cultures and retained their colony-forming capacity for a long time (for 1–36 months), the dormant forms maturating in autolyzing bacterial cultures in the presence of antibiotics acquired a nonculturable state after 1–2 months of storage. These cells remained intact and stained as viable dormant forms with the Live-Dead dye. However, they did not form colonies on a new medium under standard conditions. Research concerning their resuscitation conditions will be continued in our further work.

DISCUSSION

Of theoretical interest and practical relevance are the results of this work that contribute to our understanding of the mechanisms of short-term adaptation and long-term survival of bacterial populations in the presence of antibiotic drugs.

Our research plans included comparative analysis of the antibiotic-resistant forms that are either constitutively produced during the normal development of the model system (a submerged *E. coli* K12 culture) or generated under stress and survive in the presence of an antibiotic.

One of the conclusions drawn from the data obtained is that a bacterial culture concomitantly implements several survival strategies when exposed to bactericidal antibiotic doses. This results in the heterogeneity of the survivor subpopulation (fraction) that manifests itself at several levels. (1) The survivor fraction includes both resistant cells that grow on antibiotic-containing medium and tolerant cells which do not grow in the presence of the antibiotic. (2) The population of the first subculture on a solid medium is characterized by high phenotypic diversity that manifests itself in the coexistence of several colony morphology types. (3) The survivor fraction is heterogeneous in terms of the "life expectancy" of their colony-forming capacity. These points are to be discussed in more detail.

(1) Genotypic heterogeneity of the survivor fraction. The number of *antibiotic-resistant clones* that were detected in the survivor fraction correlated with their amount in the initial culture. The maximum percentage of antibiotic-resistant (AR) cells (CFU/mL) growing on the antibiotic-containing medium was 0.005% of the number of cells growing on the antibiotic-free medium. The latter chiefly represented *antibiotic-tolerant persister cells*; their percentage in the population at the time of antibiotic treatment was 0.01–0.001 %. Therefore, the number of antibiotic-resistant cells was quite low in the developing culture of *E. coli* K12. While the number of AR cells increases during cultivation, their percentage in the population does not change, i.e., they only form as a result of reproduction. As for antibiotic-tolerant (AT) cells, both their number and percentage increase during cultivation (Table 1). This is consistent with the generally accepted idea that persisters result from cell differentiation that involves transition from the "replicating" phenotype to the one tolerant to destructive factors, while their share is increasing in an aging culture (Lewis, 2010; Kint et al., 2012; Balaban et al., 2013). In our opinion, the fact that persister formation frequency is age-dependent, which was established in a number of works, disagrees with the idea that the formation of antibiotic-tolerant cells is a stochastic process (Lewis, 2010; Kint et al., 2012; Balaban et al., 2013). We suggest that *persister formation is a result of pre-programmed stress in an aging batch culture rather than a stochastic process*. The stress is due to nutrient depletion as well as to accumulation of the metabolic products in the medium, including intercellular communication factors that perform signal functions. They are exemplified by QS autoinducers (Struss et al., 2013) and density-dependent autoregulators such as alkylresorcinols (El'-Registan et al., 2007; Bukharin et al., 2005). This suggestion is not at variance with the following findings: (i) the stimulatory influence of programmed stress (nitrogen limitation) on persister formation (Nguyen et al., 2011); (ii) age-dependent colony morphology changes, a different type of phenotypic transition that was originally described by Krasil'nikov as "age-related variability" (El'-Registan et al., 2006); and (iii) a lack of persister cells in the "permanently young" cultures that are maintained by frequent transfers of the early exponential stage cultures (Allison et al., 2011). This hypothesis is supported by the data that SOS response genes (Grant and Hung, 2013; Allison et al., 2011) and

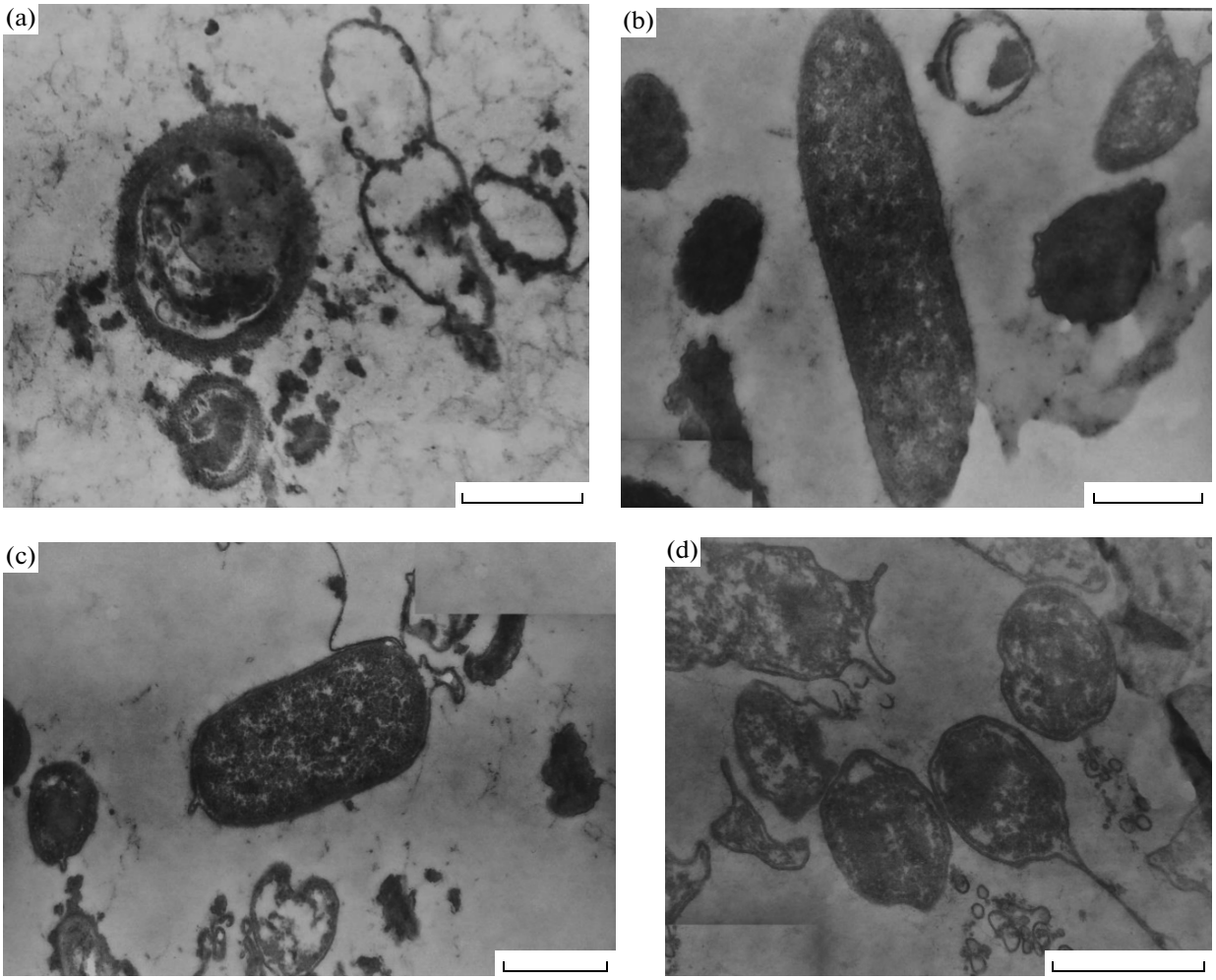


Fig. 4. Electron micrographs of *E. coli* K12 persister cells that survived for 3 months after the addition of Amp (100 μg/mL) after 4 h (a) and 7 h (b, c) of cultivation and of Cip (100 μg/mL) after 4 h of cultivation (d). The following morphotypes were revealed: (1) with a thickened envelope and a clumpy cytoplasm texture (a); (2) with a less thick envelope and a grainy cytoplasm texture (b, c); and (3) with a clumpy cytoplasm texture, a prominent periplasmic space and the presence of pili-forming cells (d). The remnants of lysed cells, "sheaths", are present in all parts of the image. Scale bar, 0.5 μm.

RpoS regulon genes (Schellhorn, 2014) are involved in producing the persister phenotype

Our studies with *E. coli* K12 revealed that environmental stressors that cause an unprogrammed stress exert a regulatory influence on the development of cell tolerance to antibiotics. Sublethal stress exemplified by heat shock (2 h; 45°C) preadapts the cells in a population to the subsequent addition of drugs by turning on the mechanisms of *metabolic adaptation to a "stressing" environment*, blocking the interaction with the environment, and temporarily stopping cell division. However, this does not imply growth arrest: after the heat shock is relieved, the Amp-untreated cultures resume their growth and catch up with the control system (without stress and Amp) in terms of cell density during the stationary phase. Although the population in the experimental system starts dying after the cessation of the heat shock and addition of the antibiotic, cell death proceeds at a lower rate. Moreover, a significantly larger number of persister cells are detectable in this system. They remain viable for a long period of time (1–3 and more months) under growth arrest conditions (in a liquid medium at 28°C in the presence of a toxicant—an antibiotic) (Fig. 3). This constitutes the principal difference between the two mechanisms of antibiotic tolerance, i.e., metabolic adaptation and transition to the persister phenotype.

The experiments in which the interval between the addition of the stressor (AR) and the antibiotic was shortened to 30 min demonstrated that the antimicrobial effect of the antibiotic was enhanced not only in terms of cell death rate but also with respect to the decrease in the number of long-term persisters (Table 4).

Therefore, analysis of the changes in survivor number in bacterial populations that were first exposed to stress and thereafter treated with an antibiotic demon-

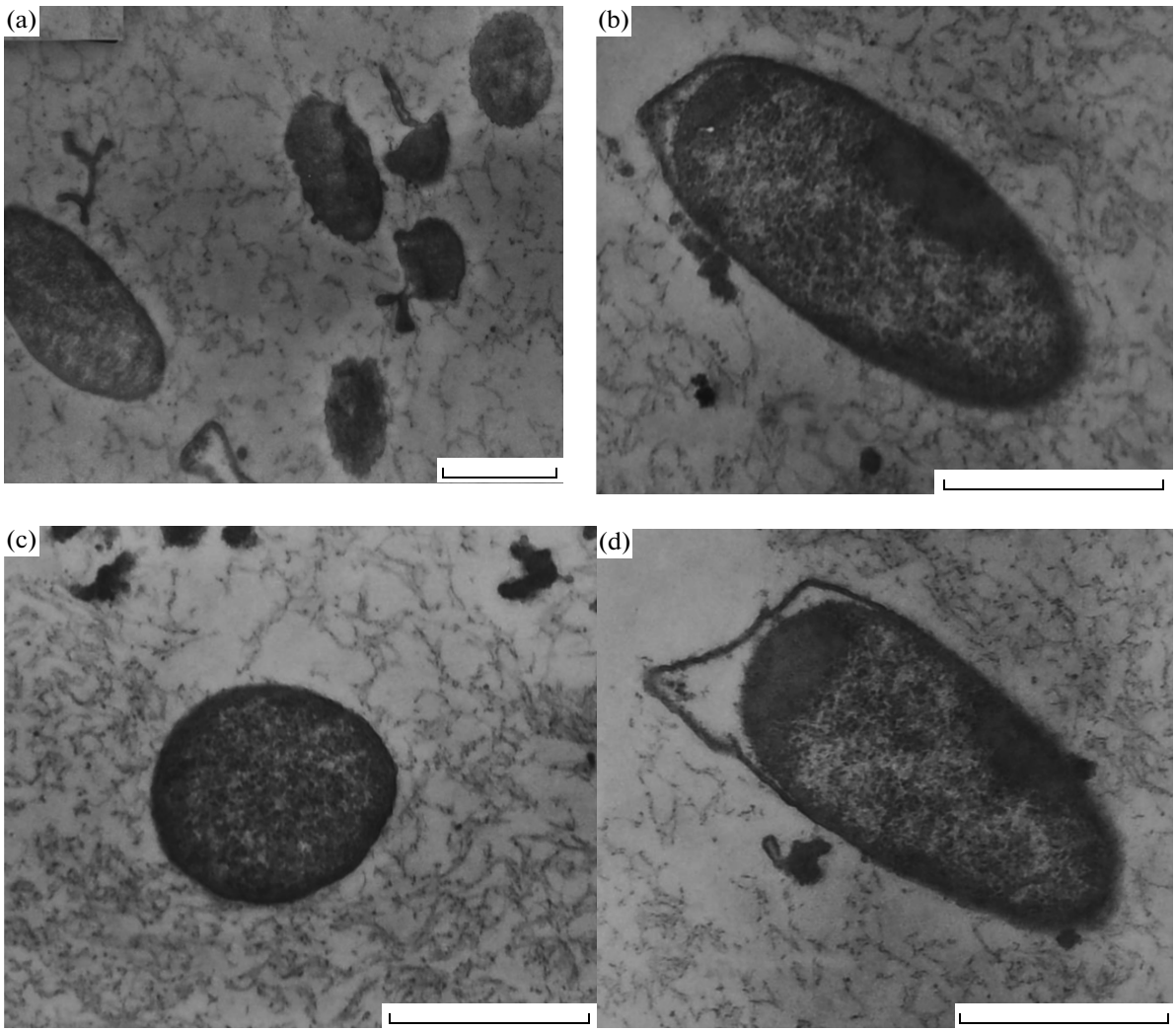


Fig. 5. Electron micrographs of *E. coli* K12 persister cells that survived for 1 month after a heat shock (45°C, 2 h) (a) and the subsequent addition of Amp (100 μg/mL) (b, c, d). All cells are characterized by a granular cytoplasm texture and an uneven distribution of cell wall material deposits in the cytoplasm. The remnants of lysed cells ("sheaths") are almost lacking, and flaky structures possibly made up of the biopolymers of lysed cells are located in the background. Scale bar, 0.5 μm.

strated that the length of the interval between the two events is of considerable importance. This is a new phenomenon revealed by us in terms of the regulation of persister formation. The period of time that is sufficient for the stress response stimulates the development of adaptive antibiotic tolerance and the increase in persister cell number. If the interval between the two events is less than the time required for the stress response to develop, cell death in the population is promoted and the number of long-term persisters is decreased, which is particularly manifest in long-term experiments (1–2 weeks).

It follows that a new parameter, the duration of viability retention (that enables population reproduction) under growth arrest conditions, should be taken into account in defining persisters as antibiotic-tolerant forms.

(2) Phenotypic heterogeneity of the fraction (subpopulation) surviving in the presence of an antibiotic was determined from its diversity in terms of colony morphology. It manifested itself in the first transfer if agar-containing standard medium (LB or LB/2) or the medium with the antibiotic (10 μg/mL Amp) were used for plating. Upon plating of the survivor fraction, which was overwhelmingly composed of persisters, a significant enlargement of the phase variant spectrum of the resulting population occurred. Apart from the dominant S variant, the colonies of the transparent (Pl), small colonies-forming (Sm), and microcolonies-forming (SCV) phenotypes developed. Predominance of the Sm phenotype was revealed by us earlier during the germination of the CLC of non-spore-forming bacteria of a large number of species (Kryazhevskikh et al., 2013; Mulyukin et al., 2008a; Pogorelova et al., 2009). This phenotype is unstable. In

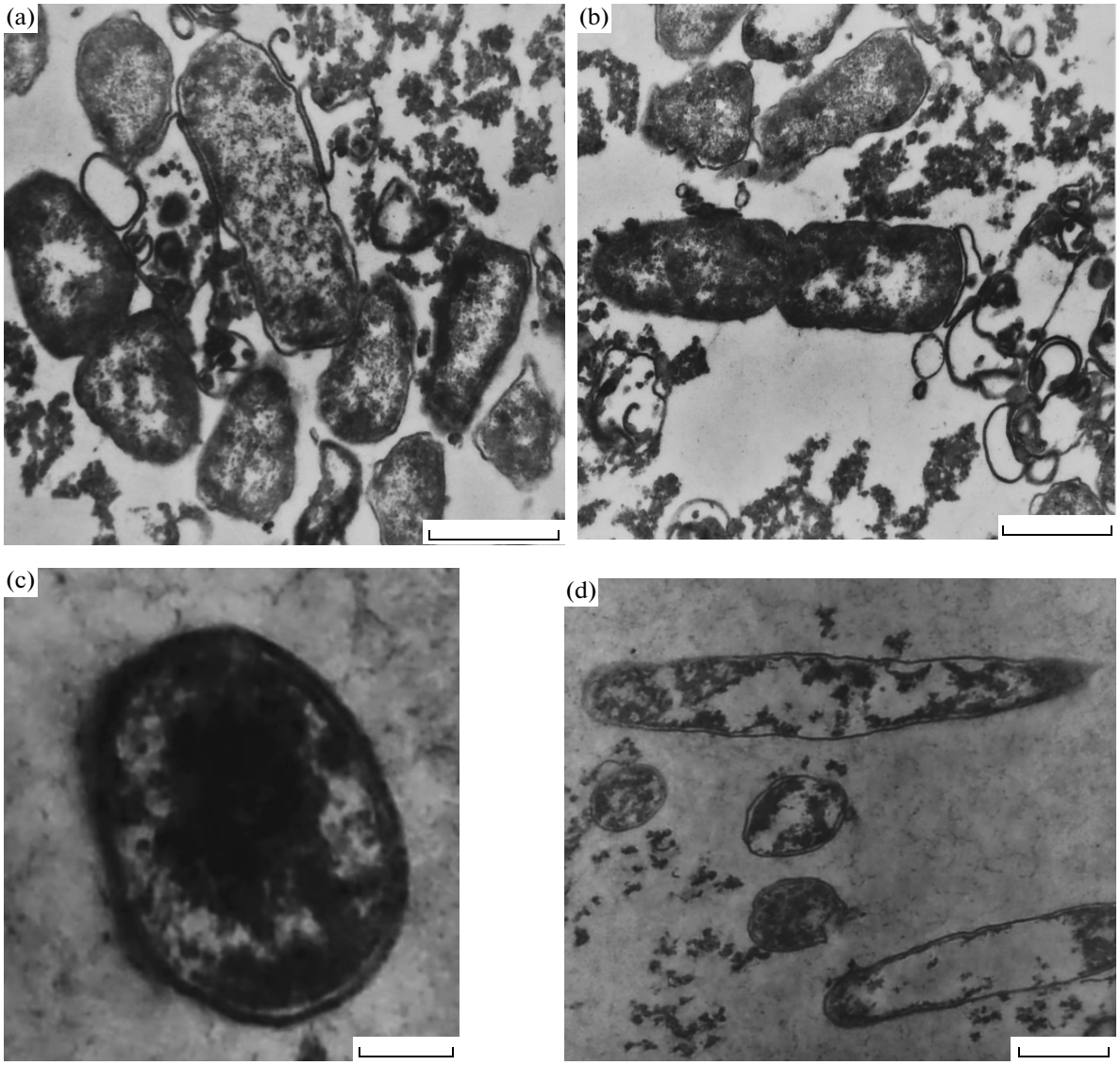


Fig. 6. Electron micrographs of *E. coli* K12 persister cells that survived in the cultures prestressed with C12-AHB (2×10^{-4} M, 30 min) with the subsequent addition of Amp (100 μg/mL) (1 month, a and b) or Cip (100 μg/mL) (3 months, c and d). The cells are characterized by a clumpy cytoplasm texture (a, b, c), detachment of the outer membrane (a, b), and a prominent periplasmic space (c). In variant (d), slowly lysing cells are present. Scale bar, 0.5 μm in a, b, and d; 0.1 μm in c.

the first transfer, it reverts to the parental dominant type or to another colony morphology variant (Kryazhevskikh et al., 2013). The SCV phenotype is antibiotic-tolerant due to its low growth rate. It was described as the antibiotic-tolerant reversible double mutant of opportunistic pathogens (Grant and Hung, 2013). SCV phenotype cells that are detected inside host cells are considered as bacterial persistence forms (Grant and Hung, 2013; Proctor et al., 2006).

Population survival implies not only the retention of cell integrity despite the influence of detrimental factors but also the capacity to resume growth (colony unit formation) under new environmental conditions, as well as the development of diverse colony morphology types that differ in terms of nutrient requirements and stress tolerance. This substantially increases the species survival potential and provides for its successful spreading. As far as opportunistic pathogens are concerned, actualization of the dissociative potential of their cells that manifests itself particularly actively during persister germination also secures their survival during the part of their life-cycle that is aimed at changing the host organism.

(3) An additional level of heterogeneity of the subpopulation surviving after the addition of the antibiotic is based on the differences among the cells with respect to the time period within which they retain viability (CFU-forming capacity). The rapid decrease in viable cell number (taking only 4 h) in the presence of the antibiotic reflects the death of the bulk of antibiotic-

sensitive cells in the population (Fig. 2, the CFU level drops from 7×10^8 to 8×10^4 cells/mL). In contrast, the subsequent decrease in CFU number (continuing for 3–7 days) is due to the gradual death of persisters. This biphasic curve of cell death in antibiotic-treated populations was demonstrated in a large number of works with all tested microorganisms (Keren et al., 2004). The decrease in the CFU-forming capacity of persister cells is due, first, to their "biochemical" heterogeneity and, second, to the instability of their metabolic state. We argue that persister cells are precursors of the dormant long-term survivors (such as CLC). They have undergone the cell differentiation stage involving molecular genetics-related events. The next stage should be transition to the anabiotic state which is based on the physicochemical processes that involve changes in membrane microviscosity, ion transport, cytoplasm dehydration, and the conformational modification of proteins and other cell biopolymers (El'-Registan et al., 2006; Bukharin et al., 2005). The hypothesis advanced in this work contributes to our understanding of the functional role of persister cells. Apart from their tolerance to drugs, they are aimed at enabling a population to survive and reproduce despite the effects of various detrimental environmental factors and, therefore, at securing the conservation of the species involved.

Electron microscopic studies with persister cells (Fig. 4–6) revealed their visual similarity to the bacterial CDC that were described by us earlier in representatives of many taxa, including gram-negative bacteria of the genera *Pseudomonas* (Mulyukin et al., 2008b) and *Azospirillum* (Mulyukin et al., 2009b). However, survivors existed under challenging conditions in our experiments, including the growth medium, the temperature (28°C), and the presence of an antibiotic as a stressor. This could induce the development of a "nonculturable" state after 1–2 months of storage. Special resuscitation techniques are required to enable these dormant forms to revert to active growth. The "nonculturable" state was described for *Micrococcus luteus*; its culture also existed under challenging conditions, for 1–3 months of incubation under chemostat conditions with an extremely low flow rate (Mulyukin et al., 2009a). Resuscitation of such dormant nonculturable forms involves protein Rpf factors that are synthesized by the organism to be resuscitated. They are produced during the lag phase of submerged cultures (Mulyukin et al., 2009a). Our further research will be aimed at testing our hypothesis that persister cells and CLC are identical in terms of molecular genetics.

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