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# Tolerance to Antimicrobial Agents and Persistence of *Escherichia coli* and Cyanobacteria

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**Abstract**—Bacterial persistence is the tolerance of a small part of a cell population to bactericidal agents, which is attained by a suppression of important cell functions and subsequent deceleration or cessation of cell division. The growth rate is the decisive factor in the transition of the cells to the persister state. A comparative study of quickly growing *Escherichia coli* K-12 strain MC 4100 and cyanobacteria *Synechocystis* sp. PCC 6803 and *Anabaena variabilis* ATCC 29413 growing slowly was performed. The cyanobacterial cells, like *E. coli* cells, differed in sensitivity to antimicrobial substances depending on the growth phase. Carbenicillin inhibiting the synthesis of peptidoglycan, a component of the bacterial cell wall, and lincomycin inhibiting the protein synthesis gave rise to nucleoid decay in cells from exponential cultures of *Synechocystis* 6803 and did not influence the nucleoids in cells from stationary cultures. Carbenicillin suppressed the growth of exponential cultures and had no effect on cyanobacterial stationary cultures. A suppression of *Synechocystis* 6803 growth in the exponential phase by lincomycin was stronger than in the stationary phase. Similar data were obtained with cyanobacterial cells under the action of $H_2O_2$ or menadione, an inducer of reactive oxygen species production. Slowly growing cyanobacteria were similar to quickly growing *E. coli* in their characteristics. Persistence is a characteristic feature of cyanobacteria.

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*Key words*: persistence, tolerance, antibiotics, carbenicillin, lincomycin, reactive oxygen species, *E. coli*, cyanobacteria

Persistence in bacteria—survival of a small part of cell population in presence of bactericidal agents—was first described in staphylococci exposed to penicillin [1]. The survival occurs due to switching-out of important cellular functions resulting in deceleration or cessation of cell division [2, 3]. Persistence is not the drug-resistance of bacteria associated with modification (via mutations) of a target for antimicrobial substance, destruction of this substance, suppression of its transport into the cells, or its excretion from the cells, which block the binding of the antimicrobial agent with its target (Scheme).

The normal cells die, because the antimicrobial agent, when bound to the target, affects its function. This leads to appearance of lethal products. For example, streptomycin (an aminoglycoside antibiotic) affects translation, thus leading to accumulation of modified cytotoxic peptides. Unlike resistance, tolerance to antibiotics is realized via switching-out of the target, thus preventing their lethal action, rather than at the level of their binding [2-4]. Antibiotic binds to the blocked target, but cannot break it. Either translation or replication cessation switches off many cellular functions, thus depriving antibiotics of their targets. This explains both tolerance of persisting cells to antibiotics and their multiple drug resistance.

This kind cells continuously appear in population, do not propagate, but do not die from bactericidal agents [2, 4]. Persisters are not mutants: they are phenotypical variants of the wild strain, which on passage give a population consisting of the great bulk of common cells sensitive to antimicrobial agent and small amount of new tolerant cells.

High tolerance to pharmaceuticals is characteristic feature of microbial films (biofilms) – a cause of chronic intractable maladies. Bacterial cells from exponential and stationary phases of periodical cultures were compared with the cells from biofilms [2, 4-6]. More persisting cells with higher tolerance were found in stationary cultures

*Abbreviations*: DAPI) 4′,6-diamidino-2-phenylindole; PI) propidium iodide.

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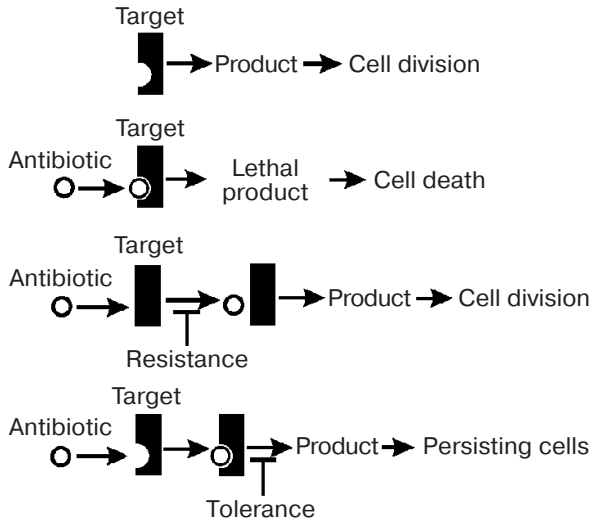


Sensitivity, resistance, and tolerance to antibiotics and persistence in bacteria (by the data of [2-4])

compared with biofilms. Stationary cultures do not practically grow, whereas the cells from biofilms slowly propagate. Early exponential cultures of *Escherichia coli*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* did not virtually contain persisters, whose concentration increased in the stationary growth phase. Multiple passages of *E. coli* in early exponential growth phase led to disappearance of persisting cells [6]. Since the cells quickly divide in the exponential phase, pass through the whole cell cycle, and do not form persisters, these data show that the persisting cells do not belong to a distinct phase of cell growth [2-4], as it was expected earlier [1, 7, 8]. These data also show that appearance of persisting cells is not a response to antibiotics, because the cells in early exponential phase do not form persisters upon the treatment with antibiotics. Addition of a culture medium corresponding to the stationary growth phase to the cells from the early exponential phase did not lead to increase in amount of persisting cells. Hence, appearance of persisters is not associated with the quorum sense effect, i.e. cell–cell communications depending on signaling substances. Persisters are the cells, which immolate their propagation to survival of the population under lethal condition [2].

The switching-out of the antibiotic targets in tolerant persisting cells is achieved by dint of specific genetic system—chromosomal toxin–antitoxin modules [2-4]. These modules are composed of two genes, which protein products are a stable toxin and coexpressed labile antitoxin neutralizing the effect of toxin due to formation of a complex with it [9, 10]. Lability and quick decomposition of antitoxin determine a probability of appearance of toxin, which is not detoxified by the antitoxin. So, the cells need in ongoing influx of antitoxin and habituate to it as to a drug (from here the term is "addiction module"). Toxin binding to the antibiotic target and inhibiting its function leads to a tolerance development [2]. In *E. coli*, a toxin (named mazF) is an mRNA endoribonuclease initiating a programmed cell death in response to various influences [11].

Appearance of persisters was found both in prokaryotes (in Gram-negative and Gram-positive bacteria) and eukaryotes (yeasts) [3]. Mainly, they are infectious agents. Persisting yeasts, as it was demonstrated on *Candida albicans*, only appear in biofilms, rather than in liquid cultures [12].

A question arises on distribution of persistence in microbial world. A comparison of various bacterial species with high and low growth rate is of particular interest. Judging from different sensitivity of exponential and stationary bacterial cultures to antibiotics [2, 4-6], the low growth rate itself provides tolerance, due to retardation of metabolic processes.

The goal of the present work is elucidation of antibiotic resistance in *E. coli*, a standard object of this kind of studies, and in cyanobacteria, which, being compared to *E. coli*, grow essentially slower. We have examined antibiotics inhibiting the synthesis of peptidoglycan, a component of cell wall (a semisynthetic penicillin carbenicillin), and protein synthesis (lincomycin).

## MATERIALS AND METHODS

The strain *E. coli* K-12 MC 4100 used in the study was obtained from Yu. A. Nikolaev, from a collection of the Institute of Microbiology, Russian Academy of Sciences, and wild strains of *Anabaena variabilis* ATCC 29413 and *Synechocystis* sp. PCC 6803 – from a collection of the Department of Physiology of Microorganisms, Moscow State University.

*Escherichia coli* cells were grown in a liquid Luria–Bertani (LB) medium, pH 7.0, in 250-ml Erlenmeyer flasks containing 50 ml of the medium at agitation at 36°C. Cyanobacteria were grown on a BG-11 medium, pH 7.0 [13], in 750-ml Erlenmeyer flasks containing 200-250 ml of the medium at agitation at 30°C under continuous illumination with luminescence lamps (~100 $\mu E \cdot m^{-2} \cdot sec^{-1}$).

The bacterial cells from cultures in exponential and stationary growth phases were used in experiments. The cells of *A. variabilis* were harvested by centrifugation at 2000*g*, and the cells of *Synechocystis* 6803 and *E. coli* – at 3000*g* for 5 min. The cells were resuspended in corresponding culture liquid with $D_{540\ nm} \sim 0.5$, supplied with tested reagents, and incubated under illumination for the time specified in the figure legends. Then the cells were sedimented, resuspended in fresh culture medium, and incubated in a sterile 96-well plate with reagents (total volume of cell suspension in the well was 200 µl, initial value $D_{540\ nm} \sim 0.5$). Change in number of cells in the plates was measured on a vertical Multiscan Plus 314 photometer (Labsystem, USA) at 540 nm.

The state of cells in bacterial cultures was estimated using a fluorescence microscope with a fluorescence dye set LIVE/DEAD BacLight: 30 μM of propidium iodide (PI) and 6 μM of SYTO9 staining nucleic acids. PI is a dye, which does not penetrate intact membranes and only penetrates into cells with damaged membranes. Being intercalated in DNA, PI fluoresces in the red region of the spectrum. Wavelengths of PI excitation and fluorescence detection were 514 and 650-710 nm, respectively. SYTO9 is also an intercalating dye, which penetrates membranes and fluoresces in the green region of the spectrum. It stains the cells with both intact and damaged plasma membranes. Wavelengths of SYTO9 excitation and fluorescence detection were 488 and 500-530 nm, respectively. The mixture of SYTO9 and PI stains bacterial cells with intact plasma membrane in green and with damaged membrane in red. PI displaces SYTO9 from nucleic acids and quenches the green fluorescence due to intermolecular excitation energy transfer by the resonance mechanism [14]. To stain nucleoids, bacterial cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, 20-μM aqueous solution) for 20-60 min. DAPI is a fluorescence dye that penetrates into cells and binds to adenine and thymine-rich regions in minor grooves of double-stranded DNA [15]. Fluorescence of DAPI was excited with a mercury lamp using a G365 absorption filter transmitting light with $\lambda$ = 300-390 nm and detected using an LP420 absorption filter transmitting light with $\lambda > 420$ nm. Fluorescence of chlorophyll was excited using a BP530-585 filter transmitting light with $\lambda$ = 530-585 nm and detected using a LP615 filter transmitting the light with $\lambda > 615$ nm. Cell preparations treated with PI, SYTO9, and DAPI were observed under an AXIOVERT 200M microscope equipped with a LSM 510 Meta confocal unit (Carl Zeiss, Germany) in light, fluorescence, and confocal modes.

## RESULTS

As shown in Fig. 1, periodic cultures of *E. coli* and *Synechocystis* 6803 differ from each other in growth characteristics, such as continuance of lag-phase, exponential growth rate, and time of achieving stationary phase. In comparison with *E. coli*, *Synechocystis* 6803 grows significantly slower. Similar decelerated parameters of growth are inherent for another cyanobacterium, *A. variabilis*. The growth of *Synechocystis* 6803 was accompanied by simultaneous increase in the cellular levels of phycobilins and chlorophyll *a*.

The data of confocal microscopy of *E. coli* cells are presented in Fig. 2 (a and c) (see color insert). The cells were stained with the LIVE/DEAD BacLight dye set as described in [2, 4]. Living cells stained with SYTO9 are green and dead cells stained with PI are red. There are non-fluorescent cells whose DNA is decomposed. Such cells are invisible in confocal mode but become visible when the image is made using a detector of transmitted light (such a cell is marked by a black arrow in Fig. 2a and is absent in Fig. 2c). Ampicillin (a semisynthetic penicillin) caused lysis of cells from quickly growing exponential cultures of *E. coli* with a few persisting cells remaining alive; the tolerance of the stationary culture was significantly higher [2, 4]. Similar results were obtained with another semisynthetic penicillin, carbenicillin: staining with SYTO9 and PI showed that the viability of cells from the stationary cultures of *E. coli* was higher that that from exponential cultures (data not shown).

Figure 2 (e and f) shows the data on the effect of lincomycin, which inhibits protein synthesis on bacterial ribosomes. When lincomycin was taken at concentration 10 μM, most of the *E. coli* cells from exponential culture were stained with PI due to affected permeability of the plasma membrane and became unviable. When the lin-

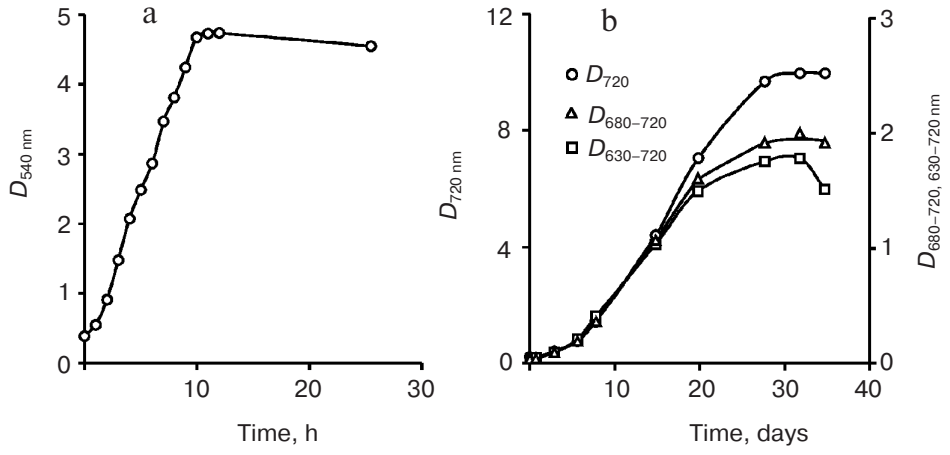


**Fig. 1.** Growth of *E. coli* (a) and *Synechocystis* sp. PCC 6803 (b) monitored by change in optical density of cell suspensions at 540 and 720 nm. The levels of chlorophyll *a* and phycocyanin in *Synechocystis* 6803 cells were monitored by the difference in optical densities $D_{680\text{-}720\ nm}$ and $D_{630\text{-}720\ nm}$.

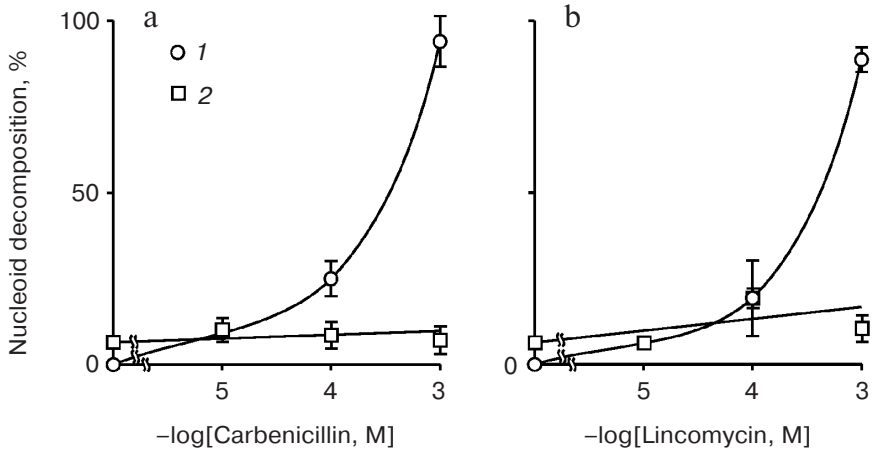


**Fig. 3.** Effect of carbenicillin (a) and lincomycin (b) on nucleoids of *Synechocystis* 6803 cells from exponential (6 day) and stationary (30 day) cultures (*1* and *2*, respectively). The cells were incubated with antibiotics for 4 h. Nucleoids were stained with DAPI.

comycin concentration was increased to 1 mM, most of the cells treated with SYTO9 and PI did not fluoresce and was only visible using the detector of transmitted light. The number of living cells stained with SYTO9 drastically increased in stationary *E. coli* cultures.

Since programmed cell death in eukaryotes manifests in destruction of the cell nucleus [16], it was interesting to retrace the state of bacterial nucleoids. We used the fluorescent dye DAPI penetrating membranes to stain the nucleoids. The fluorescing nucleoids and cells devoid of nucleoids due to effect of carbenicillin are shown on the Fig. 2 (b and d). It follows from the Fig. 2 (g and h) that the nucleoids stained with DAPI and visible against the background of fluorescent chlorophyll *a* in the *A. variabilis* cells decompose under the influence of agents impairing photosynthetic electron transport and causing apoptosis [17, 18] on treatment with NaCl, menadione, or $H_2O_2$ (Fig. 2, h and i). Nucleoid decomposition caused by menadione and $H_2O_2$ was also seen in *Synechocystis* 6803; it was more prominent in exponential than in stationary growth phase (data not shown) and was ~25 and 5% at 100 μM menadione and 30 and 10% at 5 mM $H_2O_2$ after 24 h incubation.

Nucleoids in *Synechocystis* 6803 cells from exponential cultures largely decompose with increase in concentration of carbenicillin (Fig. 3a) and lincomycin (Fig. 3b), but are retained in cells from stationary cultures. Carbenicillin at concentration $>10^{-4}$ M suppressed the growth of *Synechocystis* 6803 cells from exponential cultures and caused their lysis, but did not influence the growth of cells from stationary cultures (Fig. 4, a-d). Lincomycin suppressed the growth of *Synechocystis* 6803 cells from exponential cultures at all tested concentrations (10 μM-1 mM) and eventually caused cell lysis (Fig. 4, e and f). The lysis of *Synechocystis* 6803 cells in the stationary growth phase only occurred at lincomycin concentrations >20-30 μM (Fig. 4, g and h). Taken at concentration 10 μM, lincomycin delayed the growth of the cyanobacterium, then the growth rate increased up to the control value. The data suggest decrease in sensitivity of the cyanobacterium to carbenicillin and lincomycin on achieving stationary growth phase.

## DISCUSSION

The concentration of persisting cells in periodic cultures of *E. coli*, *S. aureus*, or *Ps. aeruginosa* in stationary growth phase was significantly higher than in exponential phase [2, 4-6]. In our experiments both the peptidoglycan synthesis inhibitor carbenicillin and the protein synthesis inhibitor lincomycin induced permeability of plasma membrane to PI and nucleoid decay in *E. coli* cells from exponential cultures, but had virtually no effect on the cells from stationary cultures.

The growth rate is a decisive factor in the transition of the cells to the persister state in which the cells become tolerant to antimicrobial agents. There are expedients in microbiological practice for transition of bacterial cultures into inactive state in which they do not grow and do not die. These expedients are used for storage of microorganisms—prolonged maintenance of their viability in conjunction with conservation of taxonomically important characteristics, including valuable characteristics of overproducers [19]. This is possible if living processes of the cells and their metabolism including genetic rearrangements are slowed. Lyophilization, cryopreservation, and other expedients are known, including the storage of cells in distilled water under petroleum jelly.

Is the tolerance to antimicrobial agents a mark of bacterial cultures with retarded growth characteristics? A comparative study of quickly growing *E. coli* and cyanobacteria *Synechocystis* 6803 and *A. variabilis* featuring slow growth was of particular interest. How would

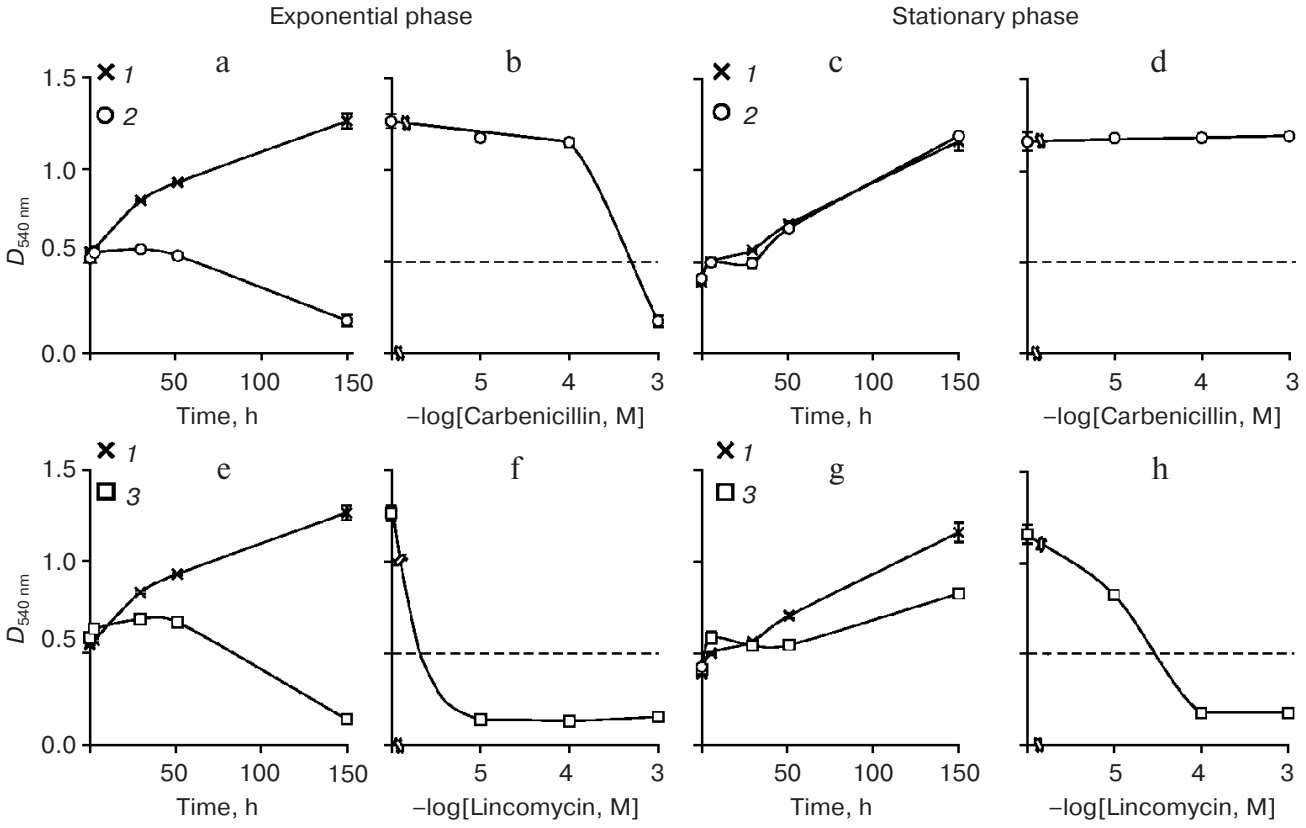


**Fig. 4.** Effect of carbenicillin (a-d) and lincomycin (e-h) on the growth of *Synechocystis* 6803 cells from exponential (14 days) and stationary (48 days) cultures: *1*) control; *2*) carbenicillin, 1 mM; *3*) lincomycin, 10 μM. Cell suspensions were diluted with culture supernatants so that $D_{540\ nm}$ was ~0.5, then either carbenicillin or lincomycin was added followed by incubation in the light for 17 h. Then the cells were centrifuged, resuspended in fresh BG-11 medium, and incubated in a sterile 96-well plate under continuous illumination. $D_{540\ nm}$ in experiments (b), (d), (f), and (h) was measured after 150 h of incubation. Dashed lines indicate the initial $D_{540\ nm}$ values before addition of antibiotics.

cyanobacterial cells growing in exponential and steady-state growth phases respond to antibiotics tested on *E. coli* cells?

Our experiments have shown that, like *E. coli*, slowly growing cyanobacteria differ in sensitivity to antimicrobial agents depending on growth phase of the cultures. Carbenicillin and lincomycin caused decomposition of nucleoids in cells from exponential *Synechocystis* 6803 cultures and had no effect on nucleoids in cells from stationary cultures. Carbenicillin suppressed the growth of exponential cultures and did not influence the growth of stationary cultures of the cyanobacterium. Suppression of *Synechocystis* 6803 growth in exponential phase by lincomycin was significantly higher in comparison with the stationary phase. A similar pattern was observed when the cyanobacterial cells were treated with hydrogen peroxide or menadione, an inducer of reactive oxygen species production. These data show that the persister cell formation in cyanobacteria depends on the culture growth phase. Exponential *Synechocystis* 6803 cultures contain little, if any, persisting cells; their number increases in stationary cultures (Figs. 3 and 4). Thus, slowly growing cyanobacteria resemble in their characteristics the quickly growing *E. coli*; persistence is inherent to free-living *Synechocystis* 6803 and *A. variabilis*. So, it is not likely that persistence is a form of symbiosis of pro- and eukaryotes characterized by lasting coexistence of organisms [20], although many infectious diseases are caused by persisting microorganisms that are commensals [21].

Since persisters are tolerant to pharmaceuticals, the question arises: how can their sensitivity to antimicrobial agents be elevated and what agent is more effective? The first response may be the transition of the persisters to metabolically active state, that is, their functions of critical importance must be set in motion to induce the cells to enter proliferation and to bring them to a state corresponding to the exponential growth phase on batch cultivation. How this task can be achieved regarding the treatment of patients is a complex question and requires further studies. The second answer is the use of reactive oxygen species, which can prove to be the most effective agents; their effects on persisting cells should be studied in more detail. The results of the present study together with the data of previous publications [17, 18, 22] are evidence for cyanobacterial sensitivity to $H_2O_2$ and menadione as an inducer of reactive oxygen species not only in

exponential, but also in stationary growth phases. The use of reactive oxygen species to damage infectious agents is rather widely used in living organisms [16]. Phagocytes in animals and humans kill infectious agents using reactive oxygen species. In plants, the hypersensitivity response often causes not only pathogens but also infected plant cells to die.

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