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EXPERIMENTAL ARTICLES

Surviving Forms in Antibiotic-Treated *Pseudomonas aeruginosa*

A. L. Mulyukin[a, 1], A. N. Kozlova[a], V. V. Sorokin[a], N. E. Suzina[b], T. A. Cherdyntseva[c], I. B. Kotova[c], A. M. Gaponov[d, e], A. V. Tutel'yan[d, e], and G. I. El'-Registan[a]

[a] *Winogradsky Institute of Microbiology, Research Center of Biotechnology, Russian Academy of Sciences, Moscow, Russia*
[b] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia*
[c] *Faculty of Biology, Lomonosov Moscow State University, Moscow, Russia*
[d] *Central Research Institute of Epidemiology, Federal Service on Customers' Rights Protection and Human Well-Being Surveillance, Moscow, Russia*
[e] *Rogachev Federal Scientific and Clinical Center for Pediatric Hematology, Oncology, and Immunology, Moscow, Russia*

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Abstract—Survival of bacterial populations treated with lethal doses of antibiotics is ensured by very small numbers of persister cells. Unlike antibiotic-resistant cells, antibiotic tolerance of persisters is not inheritable and reversible. The present work provides evidence supporting the hypothesis on transformation (maturation) of persisters of an opportunistic pathogen *Pseudomonas aeruginosa*, revealed by ciprofloxacin (CF) treatment (25–100 μg/mL), into dormant cystlike cells (CLC) and nonculturable cells (NC), as was described previously for a number of non-spore-forming bacteria. Subpopulations of type 1 and type 2 persisters, which survived antibiotic treatment and developed into dormant forms, were heterogeneous in their capacity to form colonies or microcolonies upon germination as resistance to heating at 70°C and in cell morphology. Type 1 persisters, which were formed after 1-month incubation of the stationary-phase cultures grown in the medium with decreased C and N concentrations, developed in several types of surviving cells, including those similar to CLC in cell morphology. In the course of 1-month incubation of type 2 persisters, which were formed in exponentially growing cultures, other types of surviving cells developed: immature CLC and L-forms. Unlike *P. aeruginosa* CLC formed in the control post-stationary phase cultures without antibiotic treatment, most of 1-month persisters, especially type 2 ones, were characterized by the loss of colony-forming capacity, probably due to transition into an nonculturable state with relatively high numbers of live intact cells (Live/Dead test). Another survival strategy of *P. aeruginosa* populations was ensured by a minor subpopulation of CF-tolerant and CF-resistant cells able to grow in the form of microcolonies or regular colonies of decreased size in the presence of the antibiotic. The described *P. aeruginosa* dormant forms may be responsible for persistent forms in bacteria carriers and latent infections and, together with antibiotic-resistant cells, are important as components of test systems to assay the efficiency of potential pharmaceuticals against resistant infections.

Keywords: Pseudomonas aeruginosa, survival, antibiotic-tolerant persister cells, cystlike dormant cells, antibiotic-tolerant phenotypes

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The tolerance of bacteria, especially obligate and opportunistic pathogens and clinical isolates, to antimicrobial agents is currently receiving increasing attention in view of the decreasing efficiency of antibiotics (Lewis, et al., 2007; Millar et al., 2012; Grant and Hung, 2013). Research conducted in this field provides evidence that bacterial tolerance to antibiotics, i.e., their ability to survive (but not to proliferate) in their presence, may be associated with the following events. During the development of a bacterial culture, some cells within the population acquire the proliferative dormancy state and, therefore, become insensitive to antibiotics. These cells include persisters whose cell cycle is disrupted (Bigger et al., 1944; Keren et al., 2004a; Lewis et al., 2004; Kwan et al., 2013; Wood et al., 2013) due to a phenotypic transition (Balaban et al., 2004; Gefen and Balaban, 2009) and viable nonculturable cells (Roszak and Colwell, 1987; Li et al., 2014). Apart from persister cells, antibiotic tolerance is inherent to long-term surviving cystlike dormant cells (CLC) (El'-Registan et al., 2006). Antibiotic-tolerant cells are revealed in biofilms that contain cells in various physiological states, including vegetative, metabolically quiescent, and persister cells (Costerton et al., 1999; Shah et al., 2006; Lewis et al., 2007). Importantly, the state of metabolic dormancy (anabiosis) that is typical of CLC implicates an increased phenotypic variability during their germination (El'-Registan et al., 2006). It manifests itself in colony morphology variability, including the develop-

[1] Corresponding author; e-mail: andlm@mail.ru

ment of stress-resistant variants that were detected in *Mycobacterium smegmatis, Staphylococcus aureus*, and *Corynebacterium pseudodiphtheriticum* (Mulyukin et al., 2010, 2014).

Of paramount importance for general and clinical microbiology is antibiotic tolerance/resistance of the gram-negative non-spore-forming bacterium *Pseudomonas aeruginosa*, a pathogen that causes a number of infectious diseases and is named as "the microbial hyena" (Fick, 1993). It was established that, during the life-cycle of submerged cultures of this bacterium, an extremely small subpopulation of persisters emerged that were tolerant to antibiotics including fluoroquinolones. The persister number increased during development of the culture from the exponential to the stationary growth phase (Keren et al., 2004b). The important role of the state of proliferative dormancy in terms of survival of *P. aeruginosa* is emphasized by the data on the nondividing persister cells isolated by flow cytometry of disintegrated biofilms being tolerant to an antibiotic (tobramycin) and other antimicrobial agents (silver ions) (Kim et al., 2009). Another *P. aeruginosa* survival strategy is based upon a reversible transition of its cells into a metabolically inactive nonculturable state under the influence of bactericidal agents (copper or chlorine ions) (Dwidjosiswojo et al., 2011; Bédard et al., 2014) or other stressors (freezing and thawing) (Leung et al., 1995). Inactive *P. aeruginosa* cells that lacked enzyme activities and did not express the green fluorescent protein accounted for a significant part of the biofilm population (Xu et al., 1998; Kim et al., 2009). Variants that were insensitive to specific antibiotics were also isolated from biofilms (Drenkard and Ausubel, 2002; Kirisits et al., 2005).

In terms of the general issue of *P. aeruginosa* persistence, the question to be raised concerns the subsequent fate of persister cells, their possible transition to dormant forms including CLC or other types that may enable population survival in the presence of antibiotics, and the relation between the dormant state of persisters and the appearance of antibiotic-tolerant phenotypes. Even though cystlike type dormant forms have not been documented in *P. aeruginosa*, their formation has been revealed in other representatives of the genus *Pseudomonas* (Mulyukin et al., 2008). The main approach that was earlier applied to stimulate CLC formation in pseudomonads involved the use of nutrient-imbalanced or nutrient-limited media that enhance lipid formation (El'-Registan et al., 2006). These studies can be expanded on by elucidating the relationship between two different events in *P. aeruginosa* populations, i.e., survival of persister cells in the presence of an antibiotic and formation of CLC.

The goal of the present work was to investigate the surviving forms of *P. aeruginosa* in the presence of ciprofloxacin and the relationship between their survival and phenotypic intrapopulation variation, including the emergence of antibiotic-tolerant variants.

MATERIALS AND METHODS

The subjects of this work were two strains of the gram-negative bacterium *Pseudomonas aeruginosa*: PAO1 (from the collection of the Institute of Molecular Genetics, Russian Academy of Sciences) and 393 (from the collection of the Microbiology Department of the Biology Faculty of Lomonosov Moscow State University). The *Pseudomonas* cultures were grown in Luria–Bertani (LB) broth or in a semisynthetic medium containing the following (g/L): glucose, 30; $NaNO_3$, 10; $NaH_2PO_4 \cdot 2H_2O$, 1.1; KCl, 1; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract (Difco), 5; and distilled water (pH 7.2). The inoculum that was grown overnight (for 18 h) in LB broth was added at a concentration yielding the culture optical density of 0.2 measured using a Jenway 7315 spectrophotometer (Germany) $\lambda = 450$ nm, $l = 1$ cm. The bacteria were cultivated in 250 mL flasks with 50 mL of the medium at 28°C on an orbital shaker (140–160 rpm). The semisynthetic medium was modified by decreasing the content of the inorganic nitrogen and carbon sources twofold and that of yeast extract tenfold.

Type 1 persisters (P1) of *Pseudomonas aeruginosa* that formed at the stationary phase and type 2 persisters (P2) in exponentially growing populations (according to the classification suggested by Balaban et al., 2004) were detected by challenging the cultures of the respective age to ciprofloxacin (CF) at concentrations of 5–100 μg/mL. The experimental and control cultures (with and without CF) were stored for 24 h to 1 month under static conditions at room temperature with periodic shaking. In order to determine the viability of bacterial cells, they were washed three times to remove the antibiotic (centrifugation at 7000 *g* followed by discarding the supernatant), resuspended in physiological saline (pH 7.2) according to Möker et al. (2010), and plated onto the relevant media.

The colony-forming capacity (CFU/mL) was determined by plating 10^N times-diluted cultures on agar media (1.8% agar, wt/vol) that were incubated at 28°C for 3–5 days. The number of cells that formed microcolonies (mCFU/mL) was determined by scanning the agar surface with a binocular microscope at a magnification of 2.5–5. Capacity of microcolonies to revert to the original phenotype was assessed by plating them on solid media. The most probable number of cells (MPN/mL) capable of growing in liquid media was estimated from the growth of serially diluted cultures in wells of Corning plates (50 μL of cell suspension plus 450 μL of the medium). The number of viable cells was determined using LB broth-based liquid and solid media as well as semisolid (0.3%) agar.

Heat resistance was determined from the number of cells that remained viable (based on CFU and mCFU counts) upon heating the cell suspensions at 70°C for 5 min.

The phenotypic variability of bacteria was determined based on emergence of variants that differed from the dominant type in colony size, shape, consistency, and color.

Antibiotic-resistant variants were detected on solid media with CF at concentrations of 1, 5, and 10 μg/mL, and their frequency was estimated as a percentage of the total number of colonies on the antibiotic-free medium. The isolated CF-resistant variants were consecutively plated at least three times on selective media with the same and higher CF concentrations (1–2.5 μg/mL; 2.5–5 μg/mL; 5–10 μg/mL; 10–20 μg/mL; 20–50 μg/mL; and 50–100 μg/mL).

Microscopic studies were conducted using a Zetopan (Reichert, Austria) or an Axioplan (Carl Zeiss, Germany) microscope. Bacterial cells were stained with Live/Dead Baclight kit® L-13152 (Molecular Probes) to distinguish between the live and dead cells; at least 20 fields were scanned and at least 1000 cells were monitored. Suspensions with a low cell density were concentrated 100-fold.

Electron microscopy studies. Precipitated cells were fixed in 1.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times in the same buffer, and additionally fixed with 1% OsO_4 in 0.05 M cacodylate buffer (pH 7.2) at 20°C for 3 h. After dehydration, the samples were embedded in Epon 812 epoxy resin. Ultrathin sections were contrasted for 30 min with 3% uranyl acetate solution in 70% ethanol and thereupon stained with lead citrate according to Reinolds at 20°C for 5 min. The sections were examined in a JEM-1400 electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

Statistical analysis was performed using the standard tools such as calculating the mean square deviation within a data array, applying the Student *t*-test, and establishing the correlations between two data sets. The probability criterion of $P < 0.05$ was considered sufficient for regarding the difference between data sets as significant.

RESULTS

The survivability of type 2 and 1 (P2 and P1) *P. aeruginosa* persisters was monitored under nutritional conditions of culture development, which differed from those (C/N-imbalance and N limitation) used to stimulate the formation of cystlike dormant cells (Mulyukin et al., 2010, 2014). This work mainly focuses on the long-term survival of antibiotic-treated persister subpopulations, bearing in mind that their number in *P. aeruginosa* cultures is expected to be very low (Keren et al., 2004b; Möker et al., 2010). We empirically selected the medium variants (a rich medium and a modified synthetic medium with decreased C and N content) on which the cultures developed at an identical rate (growth curves not shown) with a high biomass yield (Tables 1 and 2). The differences in nutrient concentrations could influence the persisters' survival during their long-term incubation (for 1 month and still longer periods). Under the given conditions, the number of cells in exponentially growing and stationary cultures (10^8–10^9 CFU/mL, respectively) and that of antibiotic-tolerant persisters (10^4–10^7 CFU/mL) was sufficient for microbiological research.

Formation and Survival of Type 2 Persisters

Persister formation. In exponential-phase (6–7 h) *P. aeruginosa* PAO1 cultures, the P2 number was determined by adding bactericidal doses of ciprofloxacin (CF) that killed the overwhelming majority of antibiotic-sensitive cells. On Fig. 1, typical bacterial death curves are presented. After 3 h of CF treatment (5–100 μg/mL), the P2 number varied within the (1–2) × 10^4 CFU/mL range, which accounted for 0.002–0.006% of the cell number in the culture prior to CF addition (Fig. 1a). The same P2 percentage was revealed in the exponentially growing culture of strain 393 (Fig. 1b). The P2 number and percentage of both strains were independent on the composition of either rich (Fig. 1, curves *1*) or modified (Fig. 1, curves *2*) medium. Interestingly, the P2 cells of strain 393 developing in the modified medium were less sensitive to the CF effect. The threshold concentration, above which the degree of the cell-killing effect did not depend on the antibiotic dose, was 25 μg/mL (Fig. 1b). This is in contrast to the CF effect on the persisters of strain PAO1 whose threshold concentration was 5 μg/mL (Fig. 1a).

Surviving minor P2 fractions of the strains *P. aeruginosa* PAO1 and 393 were heterogeneous in terms of tolerance/resistance to CF. The bulk fraction represented antibiotic-tolerant persisters that form colonies of the dominant S type (d = 5–10 mm) (Tables 1 and 2) after plating of washed cell suspensions on LB agar. The colonies produced by P2 persisters appeared on days 3–5, which indicated a slowdown in the division of persister cells, in contrast to the control cultures in which colony growth occurred after 24–36 h. Apart from S type colonies whose cell numbers did not exceed 10^4–10^5 CFU/mL, the LB agar-cultivated populations produced point microcolonies (mCFU) with $d \le 0.5$ mm that were difficult to detect; they were revealed during prolonged incubation (for 7–14 days or, in some cases, for up to 1 month). When the numbers of S colony-forming cells in the platings of the experimental and control cultures was above 10^5 CFU/mL, no microcolonies were revealed on LB agar. A prerequisite for their preferential development was creating selective conditions, i.e., adding CF (1 μg/mL) to the solid medium on which the dominant antibiotic-sensitive S type was eliminated. As was demonstrated on randomly chosen samples (40 mCFUs), efficiency of the reversion of microcolonies to the S type in LB agar subcultures did

Table 1. The numbers of viable cells: type 2 and 1 persisters and antibiotic-resistant (AR) (CFU/mL) and antibiotic-tolerant (AT) (mCFU/mL) variants in the control and experimental cultures of *P. aeruginosa* PAO1 that developed in the rich and the modified media 3 h after adding ciprofloxacin (CF, 25 μg/mL)

Culture	Cell number (CFU/mL) on the agar without CF (% of the cell number before adding CF)	Cell number (CFU/mL) of the AR variants on the agar with CF (1 μg/mL)	Microcolony number** (mCFU/mL) on the agar without CF	Microcolony number (mCFU/mL), AT variants on the agar with CF (1 μg/mL)**
Growth in rich medium (LB broth), exponential phase				
Control culture (7 h after inoculation) before adding CF	$(3.2 \pm 0.4) \times 10^8$ (100%)	0	0***	$(2.0 \pm 0.5) \times 10^3$ (6×10^{-4}%)
Control culture, 3 h after adding CF: the P2 subpopulation	$(2.0 \pm 0.3) \times 10^4$ (0.006%)	0	$(1.6 \pm 0.3) \times 10^4$ (0.005%)	$(5.0 \pm 0.3) \times 10^2$ (1.6×10^{-4}%)
Growth in rich medium (LB broth), the stationary phase				
Control culture (20 h after inoculation) before adding CF	$(9.0 \pm 0.8) \times 10^9$ (100%)	0	0***	$(3.2 \pm 0.8) \times 10^3$ (3×10^{-5}%)
Control culture, 3 h after adding CF: the P1 subpopulation	$(3.0 \pm 0.6) \times 10^5$ (3×10^{-3}%)	$(4.0 \pm 0.7) \times 10^1$ (10^{-7}%)	$(5.0 \pm 0.6) \times 10^4$ (2×10^{-4}%)	$(2.0 \pm 0.5) \times 10^3$ (2×10^{-5}%)
Growth and development in modified medium (with decreased C and N contents), the stationary phase				
Control culture (20 h after inoculation) before adding CF	$(7.8 \pm 0.8) \times 10^9$ (100%)	$(2.2 \pm 0.4) \times 10^2$ (3×10^{-6}%)	0***	$(3.2 \pm 0.6) \times 10^2$ (4×10^{-6}%)
Control culture, 3 h after adding CF: the P1 subpopulation	$(1.3 \pm 0.3) \times 10^7$ (0.2%)	$(1.0 \pm 0.1) \times 10^1$ (10^{-8}%)	0***	$(3.2 \pm 0.6) \times 10^2$ (4×10^{-6}%)

* The fraction of persisters and the AR and AT variants (% of the total number of viable cells before adding CF) is given in parentheses.

** In determining microcolony numbers, account was taken of the revealed 20% reversion to active growth.

*** If the macrocolony number was large, microcolonies were not detected.

not exceed 20%, and this was taken into account while estimating their numbers (Tables 1 and 2). The cells of the CF treatment-surviving fraction that were responsible for microcolony development could grow in the presence of elevated CF concentrations (5 and 10 μg/mL), and their numbers (10^2–10^3 mCFU/mL) were virtually independent on the antibiotic dose in the solid medium (Table 3). Taking account of their antibiotic tolerance, slow growth, and the ~10-fold reduced size of the colonies they formed, these cells apparently belong to the SCV (small colony variant) phenotype that was revealed in *P. aeruginosa* (Drenkard and Ausubel, 2002; review by Grant and Hung, 2013 and references therein). The fractions that remained viable 3 h after response to the antibiotic addition lacked antibiotic-resistant variants (Tables 1 and 2) that developed on LB agar with CF (1 μg/mL) in the form of colonies belonging to the S type or to the closely related Sm type with a somewhat smaller colony diameter (d = 2–5 mm).

Survival 1 month after CF treatment. Type 2 persisters that formed in exponentially growing cultures of *P. aeruginosa* PAO1 in the rich medium were characterized by a low survival rate. The numbers of the cells forming characteristic S type colonies was 4 orders of magnitude lower in survivor fractions (1 month of incubation; plots not shown). The cells of strain PAO1 that developed in the modified medium and were incubated for the same time retained the capacity for forming microcolonies but not macrocolonies whose titer decreased to zero (Fig. 2a). The numbers of culturable cells that could grow on solid or liquid media were 2–3 orders of magnitude below the total number of "live" cells (based on the Live/Dead test) (Table 4).

Table 2. The numbers of viable cells: type 2 persisters and antibiotic-resistant (AR) (CFU/mL) and antibiotic-tolerant (AT) (mCFU/mL) variants in the control and experimental cultures of *P. aeruginosa* 393 that developed in the rich and modified media 3 hrs and 1 month after adding ciprofloxacin (CF, 25 μg/mL)

Culture	Cell number (CFU/mL) on the agar without CF (% of the cell number before adding CF)*	Cell number (CFU/mL) of the AR variants on the agar with CF (1 μg/mL)	Microcolony number** (mCFU/mL) on the agar without CF	Microcolony number (mCFU/mL), AT variants, on the agar with CF (1 μg/mL)**
Growth in the rich medium (LB broth), 3 h after adding CF				
Control culture, exponential phase (7 h after inoculation) before adding CF	$(1.0 \pm 0.2) \times 10^{10}$ (100%)	0	0***	$(8.0 \pm 1.2) \times 10^2$ (8×10^{-6}%)
Control culture, 3 h after adding CF, the P2 subpopulation	$(6.0 \pm 0.7) \times 10^5$ (6×10^{-3}%)	0	0***	$(1.6 \pm 0.3) \times 10^3$ (1.6×10^{-5}%)
Growth in modified medium (with decreased C and N contents), 3 h after adding CF				
Control culture, exponential phase (7 h after inoculation) before adding CF	$(4.8 \pm 0.7) \times 10^9$ (100%)	0	0***	$(8.0 \pm 1.2) \times 10^2$ (1.6×10^{-5}%)
Control culture, 3 h after adding CF, the P2 subpopulation	$(1.1 \pm 0.1) \times 10^5$ (2×10^{-3}%)	0	0***	$(5.0 \pm 0.8) \times 10^3$ (10^{-4}%)
Growth in modified medium, 1 month after adding CF				
Control culture, exponential phase (7 h after inoculation) before adding CF	$(1.7 \pm 0.3) \times 10^9$ (100%)	0	0***	$(6.0 \pm 1.0) \times 10^1$ (10^{-6}%)
Culture with CF, surviving P2 fraction	$(6.8 \pm 0.9) \times 10^6$ (0.14%)	$(1.3 \pm 0.1) \times 10^6$ (2×10^{-2}%)	0***	$(2.4 \pm 0.4) \times 10^3$ (5×10^{-5}%)

* The fraction of persisters and the AR and AT variants (% of the total number of viable cells before adding CF) is given in parentheses.

** In determining microcolony numbers, account was taken of the revealed 20% reversion to active growth.

*** If the macrocolony number was large, microcolonies were not detected.

Dynamics of the numbers of culturable cells was independent of the CF concentration added (25 and 100 μg/mL). The P2 subpopulations that survived for 1 month contained, apart from antibiotic-tolerant cells that formed microcolonies on LB agar with or without CF, antibiotic-resistant cells (~6×10^2 cells/mL, i.e., 20% of the number of surviving culturable cells) that were not detectable 3 h after CF treatment. The presence of antibiotic-resistant variants in P2 fractions that were stored for a long time could be due to the increased number of their cells that could proliferate in the medium with the antibiotic. Enhanced survival potential was characteristic of the cells of the strain 393 subpopulation in the CF-containing medium (after 1 month of incubation). It manifested itself in retention of the capacity to form conventional S type colonies and the presence of an enlarged pool of antibiotic-resistant variants (10^6 cells/mL) (Table 2).

Formation and Survival of Type 1 Persisters

In all likelihood, the subpopulation of type 1 persisters in stationary-phase cultures is mixed (Balaban et al., 2004) and contributed by type 2 persisters.

Persister formation. In the stationary-phase (20 h) PAO1 cultures developing in the modified medium, the P1 number was 10^7 CFU/mL (0.2% of the cell number in the cultures prior to adding CF); it was 3 orders of magnitude above the P2 number in exponentially growing cultures in the same medium (Table 1). The similar dependence of the P1 number and percentage on the physiological age of the culture was revealed for strain 393 (data not shown).

The P1 numbers in stationary-phase cultures of strain PAO1 formed in the rich medium (LB broth) were lower than in the modified medium. It was 3×10^5 CFU/mL (3×10^{-3}% of the CFU titer in the cultures without CF) (Table 1). As far as strain 393 was concerned, no significant differences were revealed

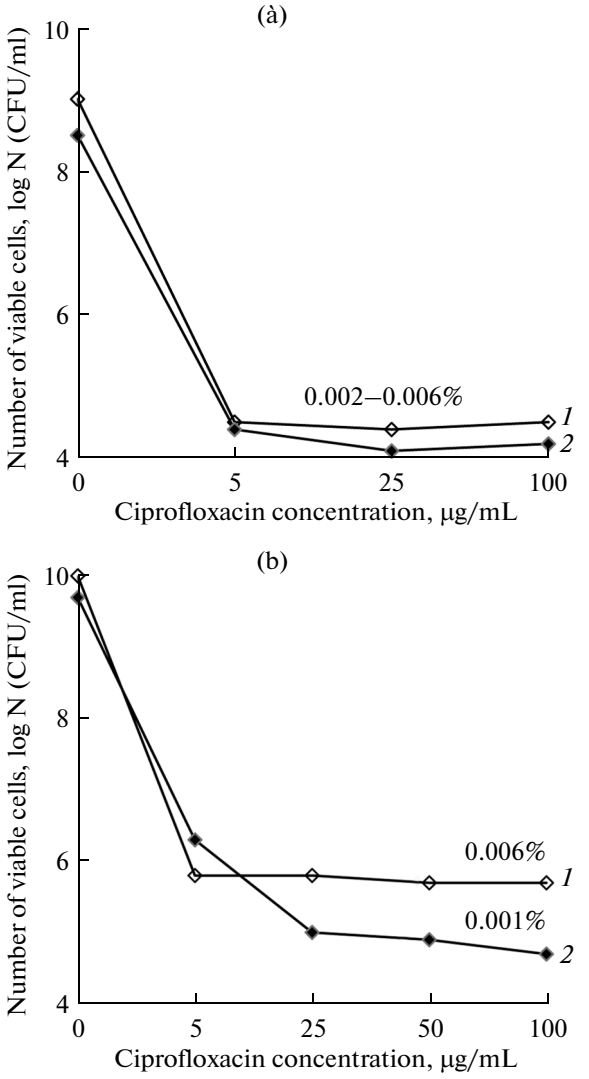

Fig. 1. Type 2 persister numbers in *P. aeruginosa* PAO1 (a) and 393 (b) cultures 3 h after adding ciprofloxacin (5–100 μg/mL). The cultures develop in the rich medium (*1*) and the modified medium with decreased C and N content (*2*). Persister fraction is shown as percent of the CFU number in the control cultures. Deviation values (5–10%) are not given.

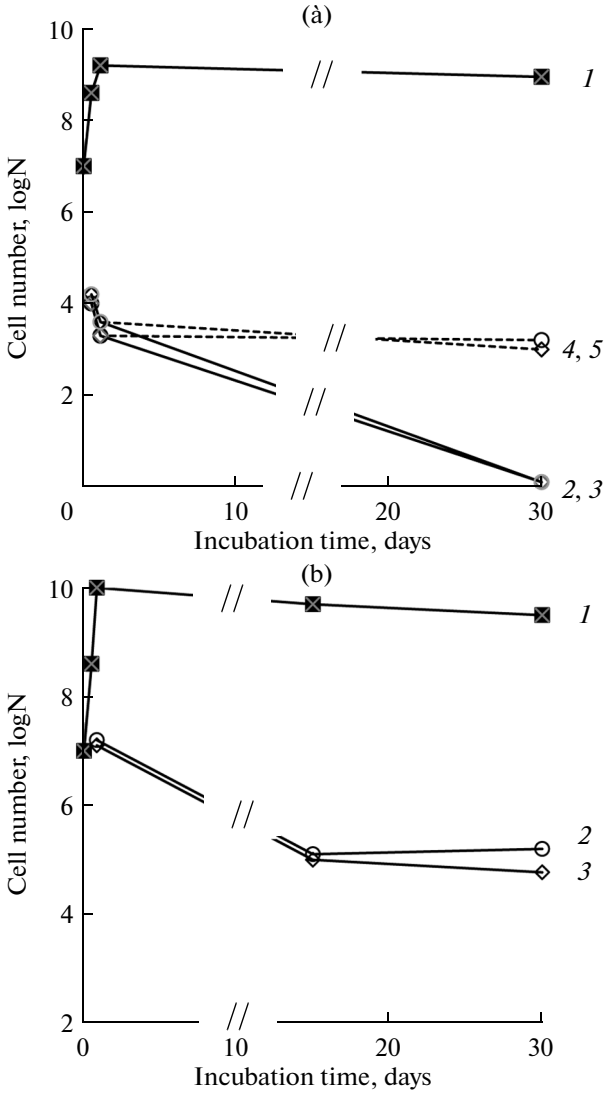

Fig. 2. Viable cell numbers in the P2 (a) and P1 (b) fractions of *P. aeruginosa* PAO1 (grown in the modified medium and stored for 1 month). Designations: control cultures (without CF), line (*1*): experimental cultures with 25 μg/mL CF, lines (*2*) and (*4*); experimental cultures with 100 μg/mL CF, lines (*3*) and (*5*). CFU numbers, full lines (*1, 2*, and *3*); mCFU numbers, dashed lines (*4* and *5*). Deviation values (5–10%) are not given.

between the P1 numbers in the rich and the modified medium (0.5 and 0.25%).

The survival of *P. aeruginosa* PAO1 cultures was secured by the presence of P1 subpopulations, along with the P2 subpopulations that formed earlier. The fraction of the cells that was viable 3 h after the CF treatment was heterogeneous. In addition to characteristic S type colonies, microcolonies of antibiotic-tolerant variants and of antibiotic-resistant Sm types developed on solid medium (LB agar with CF, 1 μg/mL). The latter variants were not detected on the media without the antibiotic, and they accounted for a minor (10^{-7}–10^{-8}%) fraction of the total cell population (Table 1). A peculiar feature of strain 393 was that the number and percentage of the slowly growing CF-resistant Sm variants revealed 15–22 days after inoculation (~10^3 CFU/mL; 10^{-6}% of the CFU number in the culture before CF addition) (Table 2) were higher than those of strain PAO1.

Survival 1 month after the CF treatment. In both strains, the P1 cells that formed in the stationary-phase cultures developing in the rich medium exhibited a low survival rate during 1 month. The CFU numbers decreased by 5–6 orders of magnitude (plots not shown). A prerequisite for long-term survival of P1 cells (as well as of P2 cells) was growing the cultures in the modified medium with decreased C and N con-

Table 3. Numbers of cells that grow in the form of microcolonies in the presence of various CF concentratioms in the control and experimental cultures of *P. aeruginosa* PAO1 and 393

Culture	Microcolony number (mCFU/mL) on LB agar with CF		
	1 μg/mL	5 μg/mL	10 μg/mL
P. aeruginosa PAO1			
Control culture, exponential phase without CF, 7 h before adding CF	$(2.0 \pm 0.5) \times 10^3$	$(6.0 \pm 0.8) \times 10^2$	$(1.0 \pm 0.3) \times 10^3$
Experimental culture, exponential phase, 3 h after adding CF (25 μg/mL), the P2 subpopulation	$(5.0 \pm 0.3) \times 10^2$	$(1.4 \pm 0.3) \times 10^2$	$(1.6 \pm 0.4) \times 10^2$
Experimental culture, stationary phase, 3 h after adding CF (25 μg/mL), the P1 subpopulation	$(2.0 \pm 0.5) \times 10^3$	$(9.2 \pm 0.9) \times 10^2$	$(4.8 \pm 0.5) \times 10^2$
P. aeruginosa 393			
Control culture, exponential phase without CF, 7 h before adding CF	$(8.0 \pm 1.2) \times 10^2$	$(1.0 \pm 0.2) \times 10^3$	$(5.0 \pm 0.7) \times 10^2$
Experimental culture, exponential phase, 3 h after adding CF (25 μg/mL), the P2 subpopulation	$(5.0 \pm 0.8) \times 10^3$	$(2.0 \pm 0.2) \times 10^3$	$(2.4 \pm 0.5) \times 10^3$
Experimental culture, exponential phase, 1 month after adding CF, the P2 subpopulation	$(2.4 \pm 0.4) \times 10^3$	$(2.4 \pm 0.4) \times 10^3$	$(1.8 \pm 0.1) \times 10^3$

tent. In this system, the titer of the cells forming conventional colonies on LB agar without the antibiotic decreased by 2–3 orders of magnitude in both strain PAO1 (Fig. 2b) and 393. Long-term (1-month) storage of the P1 fraction of strain 393 resulted in an increase in the numbers of rapidly growing CF-resistant variants of the S type, which was apparently due to cell proliferation. The antibiotic-tolerant phenotype forming microcolonies (mCFUs) on agar media with CF was also retained. Consecutive subculturing of the antibiotic-resistant variants of both strains revealed their capacity for growth on solid media in the presence of increasing CF concentrations (within the 5–20 μg/mL range). As the number of transfers increased, the Sm type colonies that dominated on LB agar with 5 and 10 μg/mL CF in the first transfer were replaced by conventional S type colonies. Only the microcolony-forming variant developed in LB agar subcultures in the presence of high CF doses (20 μg/mL).

Hence, modifying the medium composition was essential for long-term (1-month) survival of type 2 and 1 persisters.

Survival of the Control Cultures

The post-stationary control cultures (without CF) of both strains in the modified medium retained a high CFU titer for 1 month. The survival rate was particularly high in the populations of *P. aeruginosa* PAO1 whose CFU titer for a long time remained at the same level (35–45%) as that of stationary-phase cultures (Fig. 2, b). The ratio between the number of culturable cells (determined from the CFU titer) and the total number of living cells (based on the Live/Dead test) was 63% (Table 4) in 1-month control cultures. The number of antibiotic-tolerant cells that could grow in the form of microcolonies on the selective medium with CF (1 μg/mL and above) varied from 10^2 to 10^3 mCFU in the control cultures during the exponential and stationary growth phases; it did not depend on the culture's physiological age or the medium (Tables 1–3). The antibiotic-resistant Sm type was lacking, or its frequency was extremely low.

The Hypothesis on Conversion of Persisters into Metabolically Dormant Forms

In contrast to 1-month control cultures, the loss of colony-forming capacity in the overwhelming majority in P1 and P2 fractions stored for a long time (at least for 1 month), while the number of live cells remained high (Table 4) could be associated with their transition to the nonculturable state (profound dormancy), so that their resuscitation should require special reactivating techniques.

Populations of the P1 and P2 survivors were heterogeneous in their capacity for germination in the form of micro- or conventional colonies (Fig. 2, Tables 1, 2, and 4). Therefore, analysis of the relationship between their numbers and those of the dormant forms in the control cultures presents serious difficulties. Development of resistance to damaging factors such as heating at 70°C for 5 min, i.e., to conditions that produce a

Table 4. Total number of "live" cells (according to the Live/Dead test) that can grow on solid and liquid media in the persister subpopulations of *P. aeruginosa* PAO1 grown on the modified medium with 25 μg/mL CF added and stored for 1 month

Total number of live cells	Number of viable (culturable) cells	Culturable cell fraction (% of the total number of live cells)
	Type 2 persisters	
$(3.2 \pm 0.9) \times 10^6$ cells/mL	$(3.0 \pm 0.7) \times 10^3$ mCFU/mL, plating procedure $(1–4) \times 10^4$ cells/mL–MPN	0.1–1.3%
	Type 1 persisters	
$(2.2 \pm 0.6) \times 10^7$ cells/mL	$(4.9 \pm 0.2) \times 10^4$ CFU/mL $(4–6) \times 10^4$ cells/mL–MPN	0.22–0.27%
	Control cultures (without CF)	
$(8.0 \pm 0.6) \times 10^9$ cells/mL	$(5.0 \pm 0.2) \times 10^9$ CFU/mL	63%

lethal effect on vegetative cells of exponentially growing and stationary cultures (Figs. 3a, 3b) can be regarded as evidence that persisters acquire the state of metabolic (not only proliferative) dormancy.

For instance, after 1 month of incubation, the numbers of heat-resistant P1 and P2 cells of strain PAO1 (developing in the modified medium) increased from zero to 10^3–10^4 CFU/mL (Figs. 3a, 3b). This indicates transition to the ametabolic state that is characterized by resistance to detrimental factors. The increased number of heat-resistant dormant forms was revealed in the control cultures (Fig. 3c). The presence of the microcolony-forming cell fraction in suspensions with CF (Figs. 3a, 3b) apparently suggests that the antibiotic promotes the enhancement of the dormant state and population-level phase variation activity. The number of P1 forms after 1 month and their heat resistance (Fig. 3b) were comparable to those of the dormant forms that produce conventional colonies (CFUs) after heating (Fig. 3a). Similar data were also obtained for strain 393; the total share of heat-resistant cells (heated at 70°C for 5 min) in 1-month P1 and P2 fractions, based on the CFU and mCFU titers, varied from 4 to 36% of the number of unheated cells.

Hence, the fractions of surviving type 1 and 2 persisters in 1-month *P. aeruginosa* cultures included a subpopulation of highly resistant cells that apparently acquired the ametabolic dormant state.

The hypothesis that persisters are transformed into dormant forms was additionally supported by comparative studies on the ultrastructural organization of the cells (exemplified by strain PAO1) that survived for 1 month in the control and the experimental cultures.

Fine Structure of Dormant Forms and Surviving Persisters in 1-Month Cultures of P. aeruginosa PAO1

In control *P. aeruginosa* PAO1 cultures (incubated for 1 month in the modified medium without CF), intact cells were dominant and possessed the ultrastructure (Figs. 4c, 4d) identical to CLC of other *Pseudomonas* species (Mulyukin et al., 2008) and different from preceding vegetative cells (Figs. 4a, 4b). The cells with pronounced signs of destruction and lysis accounted for only several percent of cells in 1-month cultures. The CLC of the control variants of strain PAO1 were characterized by absent cell division signs, a thickened cell wall with a laminar structure, a clumpy texture of the cytoplasm that contained electron-transparent inclusions, and the presence of electron-dense compaction areas in the nucleoid.

The P1 fractions in the modified medium (14 days and 1 month after adding 25 μg/mL CF) were morphologically heterogeneous (Figs. 5a–5d). Cells of the first morphological type displayed the same features as CLC in the control cultures (Figs. 4c, 4d). Cells of the second type that were also regarded as cystlike cells were characterized by loop-shaped protrusions on the outer membrane and by an enlarged periplasmic space at the cell poles containing an electron-dense substance (Figs. 5a, 5d). Cells of the third type contain a fine-grained electron-dense cytoplasm in which intercellular structures were difficult to discern (Figs. 5a, 5c). Along with intact cells, we observed lysing cells that retained cell envelopes and contained a clear cytoplasm; the nucleoids showed signs of disintegration.

Most cells in P2 subpopulations in the modified medium (22 h after adding CF) were subject to destruction (Fig. 6a). The cells that remained intact (Figs. 6a–6c) and plausibly belonged to P2 were simi-

lar to the CLC of the second and third type in the P1 fraction (Fig. 5). In the P2 fractions incubated for 1 month, the very few cells that were detected among the debris were characterized by (Figs. 6d–6f) an enlarged periplasmic space filled with a low electron-density substance (Fig. 6f), a wave-like profile of the outer membrane (Fig. 6d), and an electron-dense cytoplasm with a homogenous or heterogeneous texture (Figs. 6b–6f). Another type of cell lacked the cell wall and was similar to L forms (Fig. 6e). The first CLC type was not characteristic of the P2 fractions.

The data obtained provide evidence for existence of several survival strategies of antibiotic-treated *P. aeruginosa* populations. These strategies involve (i) a pool of antibiotic-tolerant persister cells, (ii) antibiotic-tolerant variants that grow slowly forming microcolonies on the media with and without the antibiotic, and (iii) antibiotic-resistant variants that can form colonies of the conventional types or Sm colonies. According to the hypothesis put forward by us, long-term survival of populations (for 1 month and still longer periods) is ensured by conversion of persisters into the dormant cells including nonculturable cells that lose their colony-forming capacity under the standard growth conditions.

DISCUSSION

The existence of persisters in *P. aeruginosa* and other bacteria is a well-known fact. The reported dynamics of persister cell numbers and the pattern of their increase in the aging cultures of the tested *P. aeruginosa* strains are consistent with the previously obtained data concerning *P. aeruginosa* (Keren et al., 2004b; Möker et al., 2010). Decreased nutrient content in the medium provided for an increase in persister frequency in the stationary-phase culture of strain PAO1 (the data were obtained 3 h after adding CF), compared to the pattern of development in the rich medium (Table 1). This is in line with the data on the role of nitrogen limitation in promoting persister formation in *P. aeruginosa* (Nguen et al., 2011).

In this work, new data were obtained on the role of trophic conditions under which a *Pseudomonas* culture develops for the relatively long-term (1 month long) survival of persisters. A relatively good survival rate was attained by the P1 cells (a mixed subpopulation) that was formed during the growth of *P. aeruginosa* in the modified medium with decreased C and N content (Fig. 2). Interestingly, the number of CLC forming during the one-month incubation period which were capable of reverting to active growth (in terms of CFU/mL numbers) was higher in the control (CF-free) cultures than in the variants growing in the rich medium. This lends support to the hypothesis that persisters convert ("maturate") into CLC-type dormant forms. This is also in line with the fact that the ultrastructural organization of 1-month P1 cells and of the CLC from the control cultures was identical

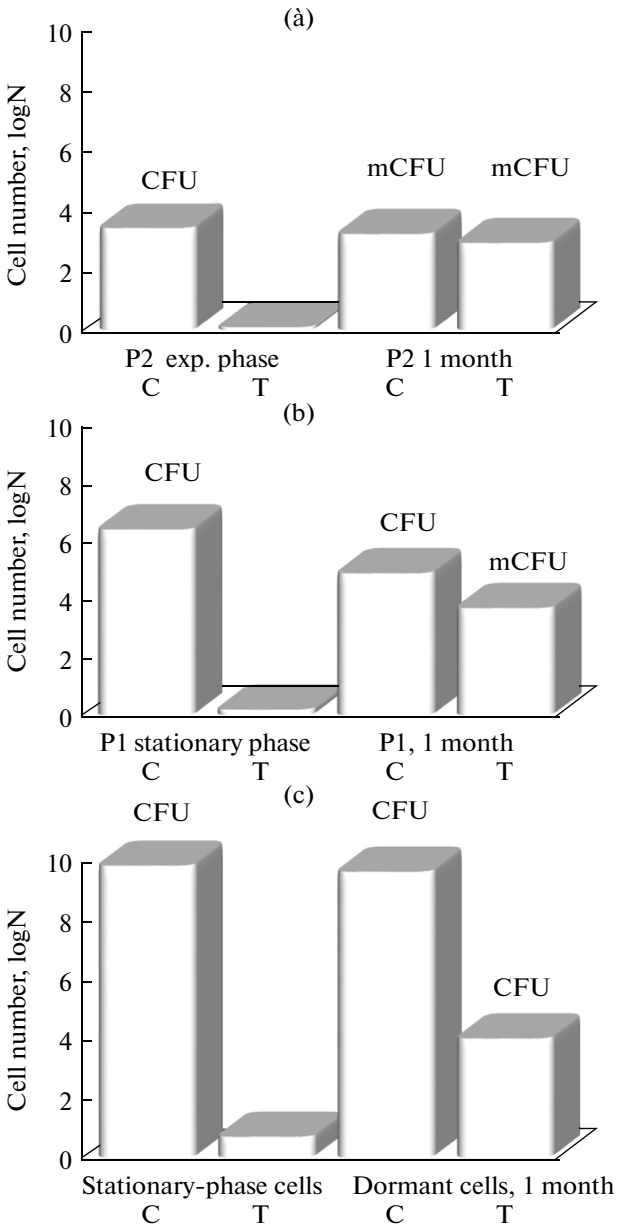

Fig. 3. Heat-resistant cell number in *P. aeruginosa* PAO1 in the P2 (a) and P2 (b) subpopulations and in cultures grown without adding CF (c) at the initial moment and after 1 month. Designations: C, unheated cultures; T, heated cultures (70°C, 5 min). Deviation values (5–10%) are not given.

(Figs. 4 and 5). It was also revealed that type 2 persisters could convert into dormant forms either belonging to the CLC type or differing from it in morphological terms (Fig. 6). Most of the P2 and P1 cells that survived for 1 month and remained intact (according to the data of the Live/Dead test) lost the ability to form microcolonies. Apparently, this was due to their transition to an nonculturable state, which was caused by the toxic effect of CF (but not by the trophic conditions), similar to the effects of other antimicrobial

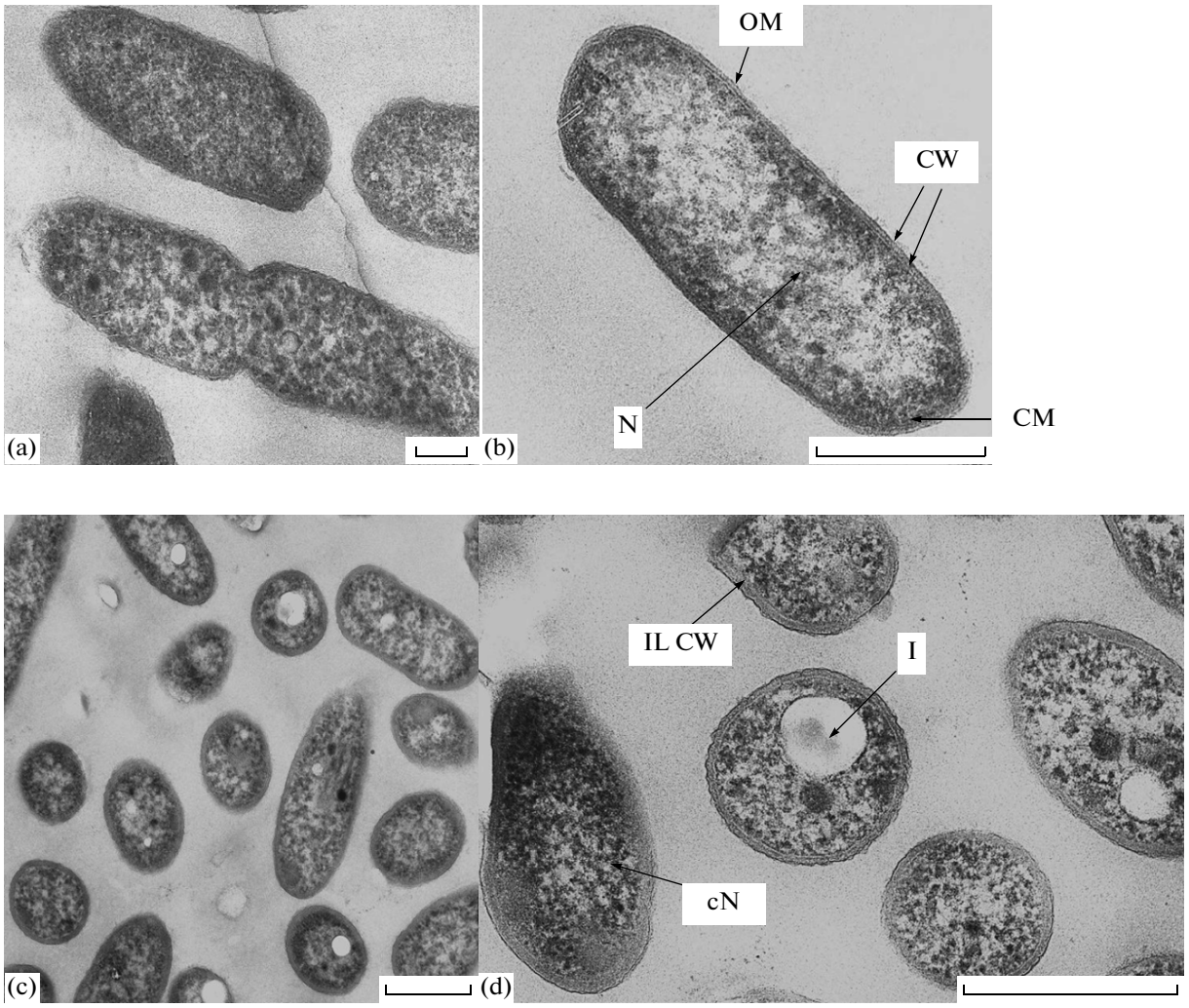

Fig. 4. Electron micrographs of the sections of *P. aeruginosa* PAO1 cells during the exponential (a) and the stationary (b) growth phase and of 1-month CLC (c, d). The culture grew in the modified medium with reduced C and N content. Designations: CW, cell wall; OM, outer membrane; IL CW, individual layers of the cell wall; CM, cytoplasmic membrane; I, inclusions; N, nucleoid; cN, compaction areas of the nucleoid. Bar, 0.5 μm.

agents such as copper or chlorine ions (Dwidjosiswojo et al., 2011; Bédard et al., 2014).

The subpopulations of cells that were generated by the "maturation" of the P2 and P1 cells of *P. aeruginosa* PAO1 1 month after the addition of the antibiotic were characterized by a larger morphological diversity (Figs. 5, 6; note the existence of several structural types, including CLC analogs and other kinds of cells, e.g., L forms) than the subpopulations of dormant cells forming in aging (1 month) cultures without CF (Fig. 4). The P2 cells of *P. aeruginosa* that survived the CF treatment in exponentially growing cultures failed to form mature type 1 CLC, in contrast to the P1 cells that survive in experimental cultures for 1 month. Therefore, it is essential that the persister subpopulations formed at various stages of the development of bacterial cultures should be pre-adapted for the subsequent transition to the dormant state; an important role should also be assigned to quorum-dependent (i.e., culture density-dependent) and other autoregulatory mechanisms. In the context of the hypothesis concerning conversion of persisters into the dormant forms, it is of relevance that the P2 numbers in logarithmic-phase *P. aeruginosa* cultures increased upon addition of *N*-(3-oxododecanoyl)-L-homoserine lactone and phenazinpyocyanine (Möker et al., 2010), the signal molecules of the quorum-sensing systems of this bacterium (Smith and Iglewski, 2003; Dietrich et al., 2006). The autoregulatory systems of pseudomonads also use factors d_1, i.e., alkylhydroxybenzenes (AHBs) (Osipov et al., 1985; Nowak-Thompson et al., 2003). Their extracellular level increases in developing bacterial cultures, and their involvement in the transition to the state of dormancy

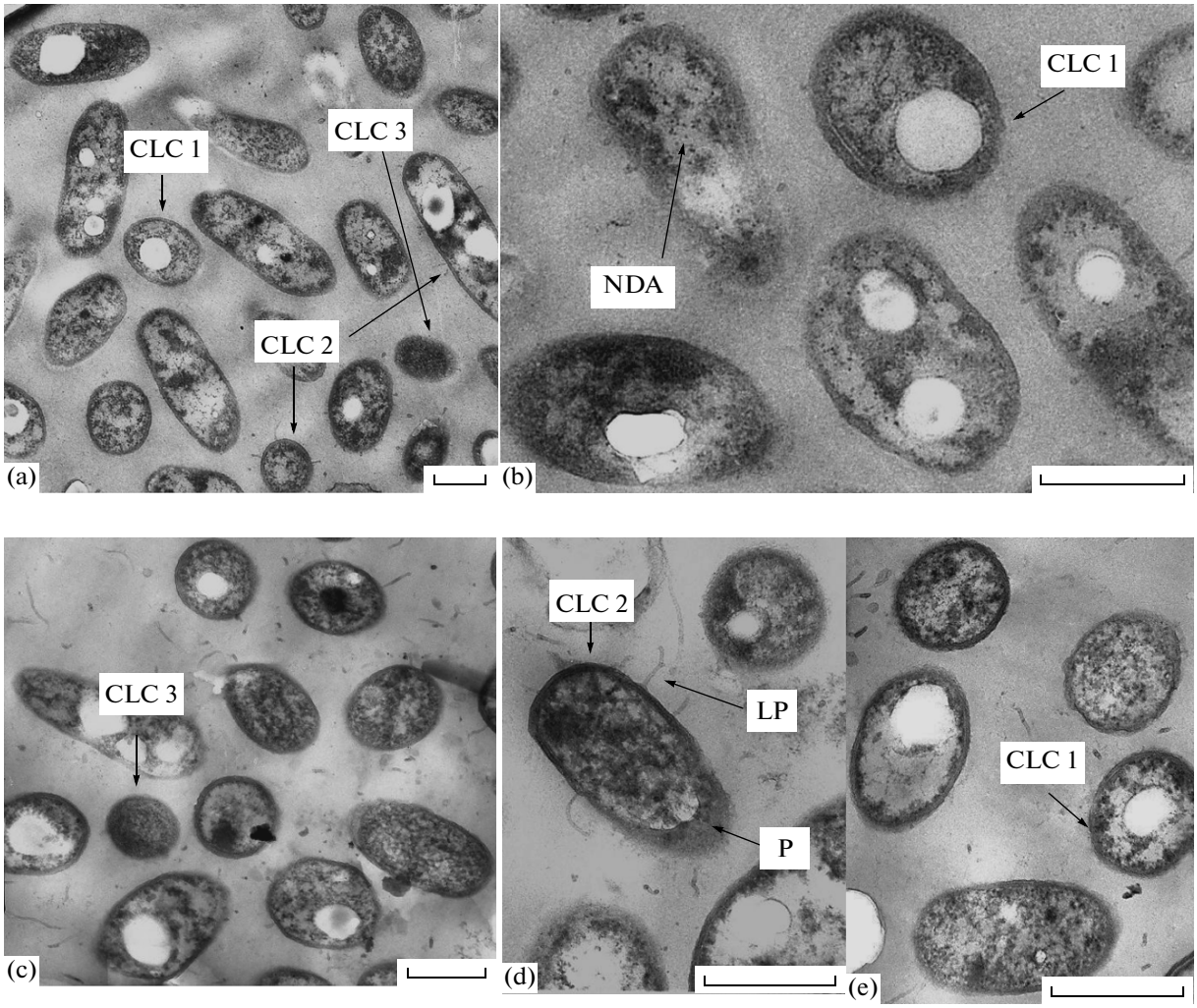

Fig. 5. Electron micrographs of the sections of *P. aeruginosa* PAO1 cells of the type 1 persister fractions 14 days (a, b) and 1 month (c–e) after adding CF. CLC of different types are shown (1–3). Designations: LP, loop-shaped protrusions: P, the cell pole with an enlarged periplasmic space containing an electron-dense substance; NDA, nucleus disintegration areas; the other designations are the same as in Fig. 4. Bar, 0.5 μm.

was established (El'-Registan et al., 2006; Mulyukin et al., 2008).

With regard to an additional survival strategy used by antibiotic-treated *P. aeruginosa*, we should emphasize the validity of the conclusion that existence of the cells in a state of proliferative dormancy in planktonic (Mulyukin et al., 2008, 2014) and biofilm (Drenkard and Ausubel, 2002; Kirisits et al., 2005) cultures is correlated with an increased emergence of anitibiotic-resistant variants. This applies to the Sm type that grows on the solid medium with CF (1 μg/mL) and is similar, in terms of colony morphology, to the kanamycin-resistant *P. aeruginosa* variant described earlier (Drenkard and Ausubel, 2002). This correlation is to a greater extent characteristic of strain 393 than of strain PAO1 (Tables 1 and 2). In addition, it was established in this work that, regardless of a culture's age and the trophic conditions of its development, the culture, as well as the P2 and P1 subpopulations, contains a fraction of CF-tolerant cells. Its cell number is relatively constant (10^2–10^3 cells/mL), and it develops after plating on an antibiotic-containing medium in the form of microcolonies ($d \leq 0.5$ mm) (Tables 1–3).

The presence of a cell subpopulation with an antibiotic-insensitive phenotype and its ability to revert to the conventional dominant type can cause serious problems in terms of medical practice.

In general, the results of this work demonstrate the multilevel heterogeneity of the type 1 and 2 persister subpopulations and of the dormant cells derived from them; this does not contradict the Yin-Yang model that takes into account the diversity of the forms and mechanisms of antibiotic tolerance (Zhang, 2014). The dormant forms of *P. aeruginosa* PAO1 revealed by

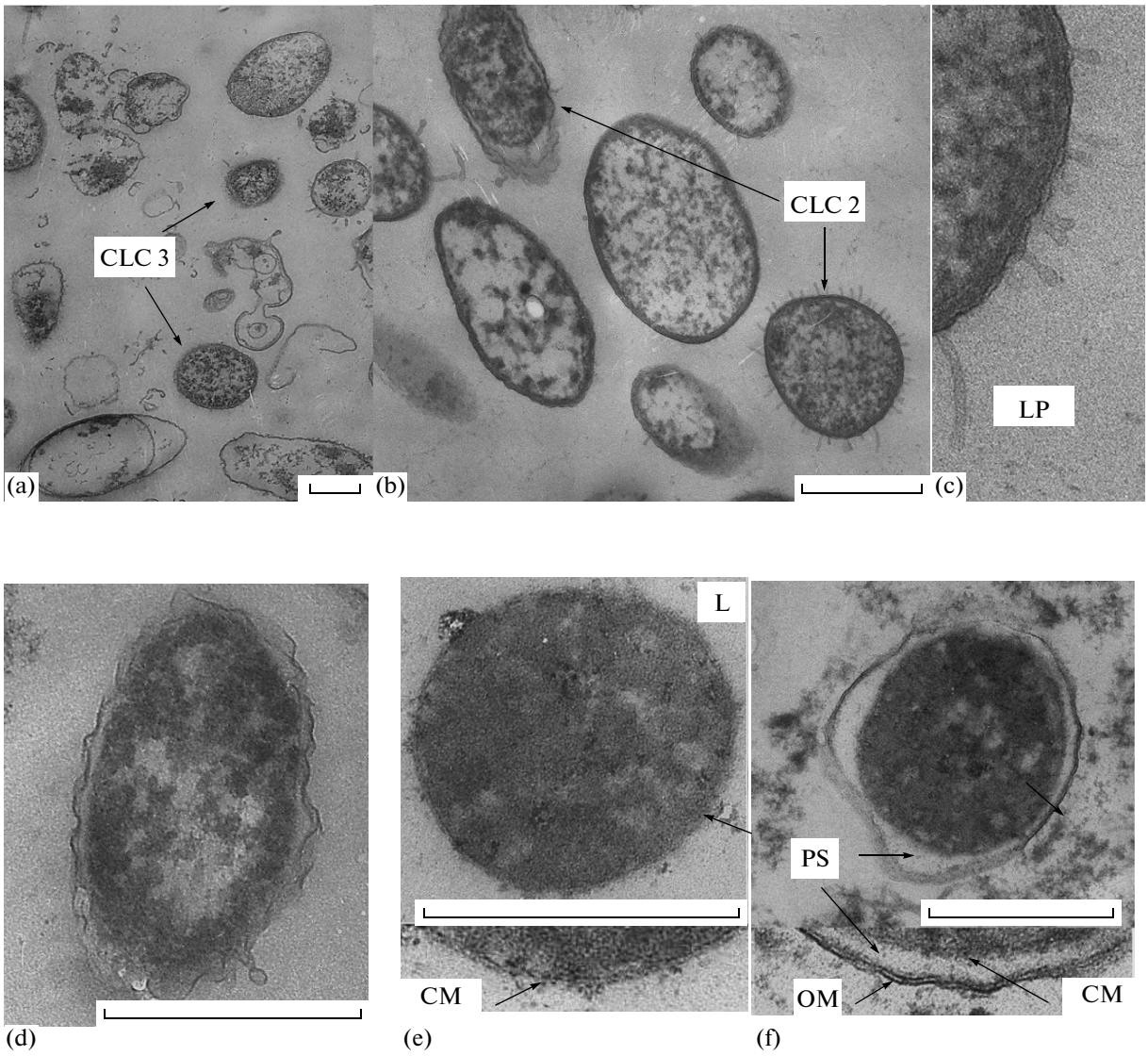

Fig. 6. Electron micrographs of the sections of *P. aeruginosa* PAO1 cells of the type 2 persister fractions 22 h (a–c) and 1 month (d, e) after adding CF. A cell fragment with loop-shaped outer membrane protrusions is shown in micrograph (c). Designations: LP, loop-shaped protrusions; OM, outer membrane; PS, periplasmic space; L, L form. Enlarged cell envelope fragments are shown in cell micrographs (e, f). Bar, 0.5 μm.

us in the control cultures and in the fractions capable of long-term survival after an antibiotic attack are candidates for the persisters that exist in bacteria-carrying individuals and during latent infections. Along with antibiotic-resistant cells, they hold much value as test system components to be used for assessing the efficiency of drug candidates against antibiotic-resistant infections.

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