ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 6, pp. 681–690. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © A.L. Mulyukin, A.N. Kozlova, G.I. El'-Registan, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 6, pp. 766–776.

EXPERIMENTAL ARTICLES

Properties of the Phenotypic Variants of *Pseudomonas aurantiaca* and *P. fluorescens*

A. L. Mulyukin[1], A. N. Kozlova, and G. I. El'-Registan

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

Received October 25, 2007

Abstract—Different capacity for phenotypic variation of *Pseudomonas aurantiaca* and *P. fluorescens* in populations of cyst-like resting cells (CRC) during their germination on solid media, was shown to be a characteristic trait of biodiversity for the dormant forms of these bacteria. This biodiversity manifests itself as qualitative and quantitative differences in the spectra and emergence frequency of phenotype variants, obtained by plating of CRC, and depends on the conditions of CRC formation and storage time. In *P. aurantiaca*, the variation was associated with transition of the wild-type S-colonial phenotype into the R-type or the more pigmented P-type. These transitions were most pronounced for the CRC obtained under nitrogen depletion (a twofold N limitation), as well as under the influence of a chemical analogue of microbial anabiosis autoinducers, C_{12}-AHB. In the latter case, the frequency of S $\longrightarrow$ R and S $\longrightarrow$ P transitions (up to 70% and 80%, respectively) depended on the C_{12}-AHB concentration (1.0×10^{-4} M and 2.5×10^{-4} M) and on the storage time of CRC suspensions (from 3 days to 1.3 months). In the CRC populations grown in nitrogen-deficient media, R-type appeared with a frequency of up to 45% after at least four months of storage. In the case of *P. fluorescens*, S $\longrightarrow$ R transitions depended not only on the storage time of CRC and C_{12}-AHB concentrations, but also on the composition of the solid medium used for plating. Differences were shown between the R-, P-, and S-variants of *P. aurantiaca* in such morphological, physiological, and biochemical characteristics as the growth rate (μ_{max}) in a poor medium, biomass yield (Y_{max}), resistance to streptomycin and tetracycline (LD_{50}), and the productivity in extracellular proteases. The R- and S-variants of *P. fluorescens* differed in their growth characteristics, resistance to high salinity and oxidative stress, as well as in their sensitivity to exogenous introduction of chemical analogues of microbial autoregulators (C_{12}-AHB and C_7-AHB). Hence, both the formation of dormant forms of the various morphological types [1] and intrapopulation phenotypic variability observed during their germination are important for the survival strategy of pseudomonads under unfavorable environmental conditions.

Key words: phenotypic variability, S $\longrightarrow$ R transition, biodiversity of resting forms, cystlike resting cells, anabiosis autoinducers, alkylhydroxybenzenes.

DOI: 10.1134/S0026261708060052

In the previous work [1], we demonstrated the formation of cyst-like resting cells (CRC) of pseudomonads, *Pseudomonas aurantiaca* and *P. fluorescens*, as well as of other non-spore-forming bacteria [2–5]. CRC possess the following, common properties of bacterial resting forms: (1) long-term preservation of viability; (2) absence of an experimentally detectable level of metabolism; (3) specific features of ultrastructural organization which point to cytodifferentiation; and (4) formation during the ontogenetic developmental cycles of microbial cultures [1–5]. The acquirement and maintenance of a dormant state is controlled by low-molecular-weight anabiosis autoinducers, d_1 factors, which in some microbial species belong to alkylhydroxybenzenes (AHB). alkylresorcinols [4, 6–8]. When dormant forms enter a new development cycle, the phenotypic (phase) variability of the microbial population often occurs, which can be easily diagnosed by the morphology of colonies developing on solid media, or by the predominance of a variant adapted to selective environmental conditions. This was previously demonstrated for various types of *Bacillus cereus* and *B. licheniformis* dormant forms (endospores, CRC, and anabiotic cells formed under the influence of AHB) [9, 10] and anabiotic cells of *Staphylococcus aureus* [11] and *Pseudomonas aurantiaca* [12].

Special attention should be paid to the intraspecies polymorphism of dormant bacterial forms, which is especially pronounced in bacilli (*B. cereus*, *B. subtilis*, and *B. lichenformis*) whose developmental cycle culminates in formation of endospores or CRC, depending on growth conditions that favor or inhibit spore formation

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

[2, 9, 10]. Importantly, morphologically different types of dormant forms (endospores, CRC, and anabiotic resting cells) of *B. cereus* vary in their ability for phenotypic variation. For instance, a wide spectrum of phenotypic variants appeared in the first transfer during germination of CRC in contrast to endospores [9]. In the case of gram-negative, non-spore-forming bacteria of the genus *Pseudomonas*, we have also demonstrated the intraspecies diversity of dormant forms. This diversity manifested itself in differences in the following physiological and morphological characteristics of pseudomonad CRC: preservation of the proliferation potential, thermal resistance, intrapopulation heterogeneity in reaction to staining with fluorescent dyes. Formation of different morphological types of resting cells depended on the cultivation conditions [1]. This work concentrates on another level of the biodiversity of pseudomonad dormant forms, that is, differences in their phenotypic variability during germination on solid media.

The goal of this work was to study the differences between the resting cells of *P. aurantiaca* and *P. fluorescens* formed under different conditions, in their ability for phenotypic variation, as well as to describe the physiological and biochemical properties of the isolated variants.

MATERIALS AND METHODS

The objects of research were gram-negative bacteria, *Pseudomonas aurantiaca* VKM B-1558 and *P. fluorescens* NCIMB 9046. Colonial R- and P-types were obtained upon plating CRC suspensions onto a solid medium. The methods used to obtain cyst-like resting cells were described in our previous work [1]. The S-variant of the collection strains was dominant in experiments to obtain CRC and to study the intrapopulation variability. The phenotype variability index was determined as the percentage of S-, R-, and P-type variants of the total colony number. The variants were selected based on their colony characteristics: shape, consistency, and pigmentation.

Media and cultivation conditions. Cultures of *P. aurantiaca* and *P. fluorescens* variants were grown in a 50% nutrient broth or balanced synthetic medium M9, as well as in a deficient synthetic medium with twofold nitrogen limitation and twenty-fold phosphorus limitation (as compared to the M9 medium). Bacteria were cultivated at 28°C in 250-ml flasks (50 ml of the medium) in a shaker at 140–160 rpm. Stationary-phase cultures of the S-, R-, and P-variants grown in 50% nutrient broth or in a nutrient broth-wort medium (1 : 1), served as inoculum; they were added to the initial optical density (OD) 0.03–0.08 (Specord, λ = 540 nm, l = 10 mm).

Microbiological methods. Growth of the cultures was monitored by OD of cell suspensions, maximum specific growth rate (μ_{max}), and maximum biomass yield (Y_{max}). To determine the number of colony-forming units (CFU), cell suspensions in appropriate tenfold dilutions were plated onto nutrient agar or nutrient agar supplemented with wort and incubated at 28°C for 3 days. Dry cell mass (DCM) was determined after 24-h drying at 105°C. Microscopic examinations were carried out using a Reichert microscope (Zetopan, Austria) equipped with a phase-contrast device.

Stress induction. Oxidative stress was caused by hydrogen peroxide (170–2000 μm); high salinity stress was induced by increasing NaCl concentration in the M9 medium from 0.05% (control; 8.5 mM) to 340 mM–1.7 M. Tetracycline or streptomycin was added simultaneously with inoculum to final concentrations of 10–1000 μg/ml. Resistance of the cultures to stress factors was assessed by changes in OD as compared to the control culture. Antibiotic resistance was monitored as the growth inhibition effect $\Delta OD_{test}/\Delta OD_{control}$ calculated as the ratio between the increase in OD in the test variant and that in the control variant after 24-h incubation at 28°C. The LD_{50} values were determined as doses of antibiotics, H_2O_2, or NaCl, which caused a 50% inhibition of the growth.

In some experiments, we assessed the **sensitivity of the cultures** to chemical analogues of bacterial growth and development autoregulators, C_7-AHB or C_{12}-AHB, added simultaneously with inoculum in concentrations ranging from 5 to 1000 mM. The AHB concentrations that caused the growth-stimulating and inhibiting effects were determined. Effects of C_7-AHB and C_{12}-AHB on bacterial growth were evaluated from $\Delta OD_{test}/\Delta OD_{conrol}$ ratios calculated for 8–24 h intervals depending on the growth characteristics of the bacteria under study.

The productivity in extracellular proteases was determined using the modified Anson's method by the rate of accumulation of casein hydrolysis products (2% solution), which were not precipitated by trichloroacetic acid and reacted with the Folin-Ciocalteu reagent, giving color at λ = 670 nm. The unit (mE) of proteolytic activity was expressed in a tyrosine equivalent which corresponds to 1 μmol (0.181 mg) of tyrosine produced upon casein hydrolysis over 1 min at 30°C.

Measurements in three independent series of experiments were repeated threefold. The presented results show the average values. The data were statistically analyzed by the Student's test; $P < 0.05$ was taken as sufficient.

RESULTS

The phenotypic variation in *P. aurantiaca* and *P. fluorescens* was determined from the number of morphologically different colonies observed at the first transfer on nutrient agar of the CRC formed in developmental cycles of the dominant S-variants of each strain. The phenotypic variability depended on the method of cyst-like resting cells production [1] and time of their stor-

Table 1. Description of the phenotypic variants of *P. aurantiaca* and *P. fluorescens* and conditions of their emergence

Type	Description	Variant of CRC obtaining	Conditions of emergence		Occurrence frequency*
			Storage time	Medium	
		P. aurantiaca			
R	Pink wrinkled colonies with uneven edges	**1**, (twofold N limitation), **2**, (C_{12}-AHB 1×10^{-4} M), **2** (C_{12}-AHB 2.5×10^{-4} M)	4 months and more 3 days 1 month and heating at 60°C, 5 min	Nutrient agar Nutrient agar Nutrient agar	45–50% 67% 100%
P	Highly pigmented pink round smooth colonies with even edges	**2** C_{12}-AHB $\times 10^{-4}$ M	1.3 months	Nutrient agar	78%
dominant S type	Pink rounded smooth colonies with even edges	**1** (twofold N limitation), **3** (SiO_2-containing medium), **4** (soil extract) and stationary-phase culture	7 days – 3 months 7 days – 3 months 7 days – 3 months	Nutrient agar Nutrient agar Nutrient agar Nutrient agar	93% 97% 98% 100%
		P. fluorescens			
R	Transparent rough or wrinkled colonies	**1** (twofold N limitation), **2** (C_{12}-AHB 1×10^{-4} M), **2** (C_{12}-AHB 2.5×10^{-4} M)	3–5 months 20 days 20 days	Nutrient agar–wort medium Nutrient agar–wort medium Nutrient agar–wort medium	52% 20% 22%
M	Cream-colored slimy colonies with raised center and even edges	**2** (C_{12}-AHB 1×10^{-4} M), stationary-phase cultures	3 days	Nutrient agar–wort medium Nutrient agar–wort medium	49% 31%
Sm Mm	Semi-transparent or pigmented small round smooth colonies with even edges (d = 1–1.5 mm)	**2** (C_{12}-AHB 1×10^{-4} M)	1.3 months	Nutrient agar–wort medium	59%
dominant S type	Transparent rounded smooth colonies with even edges (d = 6–8 mm)	**1**, **2**, **3**, **4** stationary-phase cultures	up to 3 months	Nutrient agar	97–99%

* At growth conditions and storage time were optimal for the emergence of the variants.

age (Table 1). After plating of *P. aurantiaca* CRC obtained in variant 1 (twofold N limitation, storage for more than four months), irregularly shaped (rough) wrinkled colonies (R-type) emerged at a frequency of 45–50% of the total colony number. However, this S $\longrightarrow$ R transition was not the case after plating of CRC of the same variant stored for shorter periods (3 days–3 months). R-type colonies appeared as a minor variant with a 2% frequency after inoculation of *P. aurantiaca* CRC suspensions produced by variant 4 (incubation in soil extract; 1.3 months).

However, phenotypic variation was most prominent (both in qualitative and quantitative terms) after plating of the CRC produced according to variant 2 by adding C_{12}-AHB in concentrations of 1×10^{-4} M and 2.5×10^{-4} M) [1]. The populations grown from the *P. aurantiaca* CRC of this variant contained highly pigmented colonies (P-type) as well as R-type colonies. The occurrence frequency of P- and R-variants depended on the concentration of C_{12}-AHB and the storage time of the CRC suspensions. For instance, the ratio of P-type colonies grown from the CRC obtained by addition of C_{12}-AHB (1×10^{-4} M) increased to 80% during storage for up to 40 days, while the number of S-type colonies decreased, and R-type appeared at a relatively low frequency (5–15%) (Fig. 1a). The opposite pattern was the case for the CRC obtained by adding a higher concentration of C_{12}-AHB (2.5×10^{-4} M) and stored for 3–20 days (Fig. 1b). The highest ratio of R-type colonies (45–67%) was observed after plating of CRC after three days of storage. At further incubation of these CRC for up to 40 days, the frequency of R-type occurrence decreased to 15%, while the ratio of S-type colonies increased to 60%, and that of P-type remained the same (~25%) (Fig. 1b). In the control variants obtained by plating of the stationary-phase cultures, the S $\longrightarrow$ R and S $\longrightarrow$ P transitions were not observed. Importantly, heating of the *P. aurantiaca* CRC obtained according to variant 2 (C_{12}-AHB, 1×10^{-4} M; storage for 1.3 months) at 60°C for 5 min resulted in 100% frequency of occur-

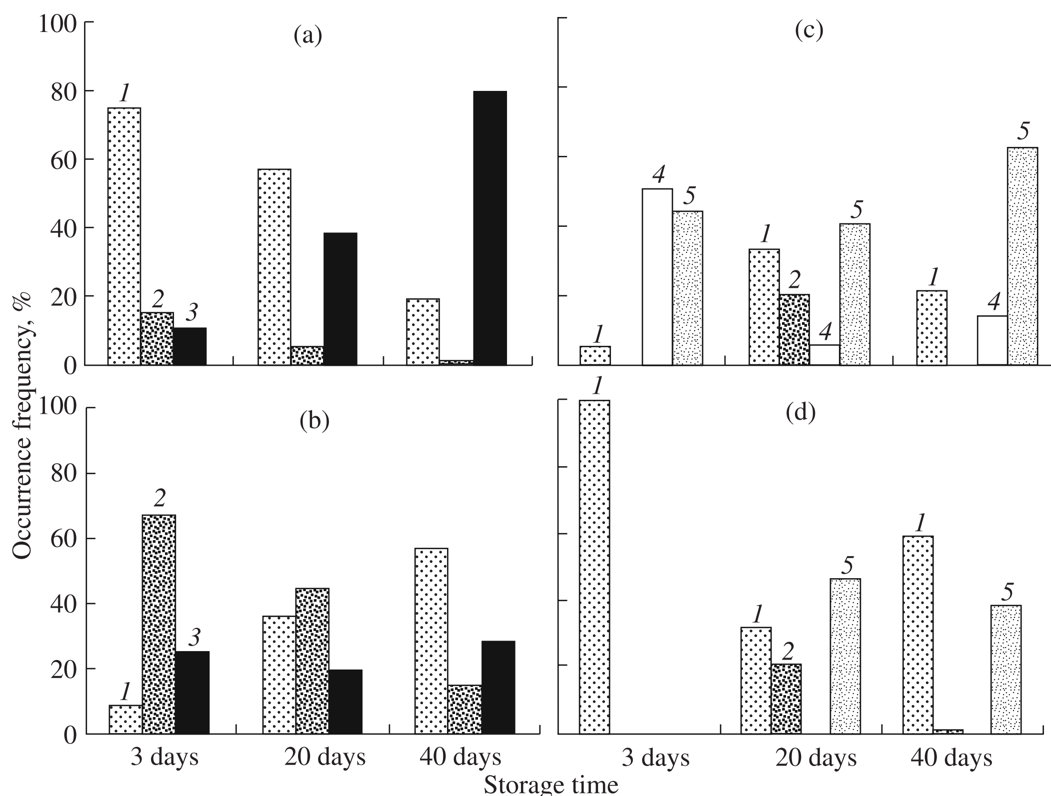

Fig. 1. Occurence frequency of the colony variants after germination of anabiotic CRC of *P. aurantiaca* (a, b) and *P. fluorescens* (c, d) obtained by addition of C_{12}-AHB at concentrations of 1×10^{-4} M (a, c) and 2.5×10^{-4} M (b, d) and stored for 3–40 days. Designations : *1*, S-type; *2,* R-type; *3*, P-type (*P. aurantiaca*); *4*, mucoid M-type (*P. fluorescens*); *5*, small Sm- and Mm-types.

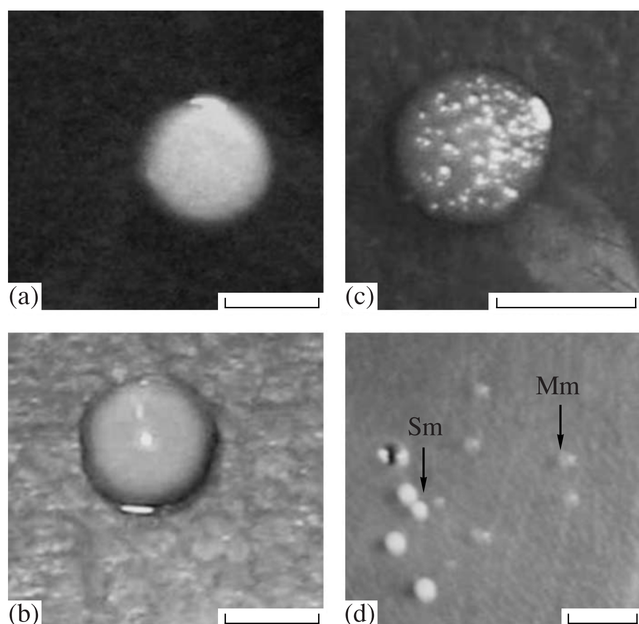

Fig. 2. Colonies of *P. fluorescens* on nutrient broth-wort (1 : 1) agar: (a) wild-type S colonies; (b) mucoid M-type; (c) R-type, and (d) small colony forming Sm- and Mm-types. Scale bar, 5 mm.

rence for R-type colonies (Table 1), although the numbers of viable cells (CFU) decreased by four orders of magnitude [1]. Heat treatment probably resulted in elimination of the S-variant and favored the predominant development of the R-type.

As for the other *Pseudomonas* species, *P. fluorescens*, the phenotypic variation depended on the conditions of CRC formation, storage period, and the composition of the solid nutrient media used for CRC inoculation. For example, the populations grown on nutrient agar from CRC of different variants were represented only by S-type colonies, i.e. phenotypic variation was not observed (Table 1). However, a wide spectrum of colony variants was observed on solid nutrient agar supplemented with wort (as an additional carbohydrate source). Plating *P. fluorescens* CRC of variant 2 (C_{12}-AHB, 1×10^{-4} M) on this medium resulted in the appearance of a high ratio (up to 50%) of mucoid (M-type) translucent creamy and slimy colonies (Fig. 2). The frequency of S $\longrightarrow$ M transitions decreased in the course of CRC maturation and was the highest (49%) in the cultures stored for three days (Table 1 and Fig. 1c). After plating of the CRC induced by high C_{12}-AHB concentrations (2.5×10^{-4} M), the M-variant did not emerge (Fig. 1d). Besides, Sm- and Mm-types, represented by small semitransparent and mucoid colonies

(Fig. 2) of about 1 mm in diameter (in contrast to other types with the colony diameter of 6–8 mm) developed in CRC populations of variant 2 (AHB addition) plated onto nutrient broth-wort agar.

The emergence of the R-variant was detected only on the nutrient agar–wort medium (1 : 1) after plating of *P. fluorescens* CRC, which were grown in a nitrogen-deficient media (variant 1) and stored for 3–5 months or were produced according to variant 2 (C_{12}-AHB, 1×10^{-4} M and 2.5×10^{-4} M) and stored for 20 days. Noticeably, the phenotypic variability of anabiotic *P. fluorescens* CRC of variant 2 (addition of C_{12}-AHB in various concentrations) depended on the duration of their subsequent storage, as it was demonstrated for *P. aurantiaca*. For example, R-variants emerged at a frequency of 20–22% upon plating *P. fluorescens* CRC, produced by treatment with C_{12}-AHB and incubated for 20 days. It was subsequently almost completely eliminated and replaced by other dominant S-type or Sm- and Mm-variants after 1.3 months of storage (Fig. 1c, 1d).

Hence, changes in the spectrum of phenotypic variants were found in the course of germination of the pseudomonad CRC formed under various cultivation conditions. The phenotypic variability depended on both the type of dormant pseudomonad forms and storage time. Such an intraspecies heterogeneity of dormant bacterial forms can be considered as a mechanism involved in the adaptation of microorganisms to new environmental conditions. Another goal of our investigation was to study the differences between *P. aurantiaca* and *P. fluorescens* variants, developing from germinating CRC, in their adaptive capabilities.

Our experiments demonstrated that, when grown in a rich medium (50% nutrient broth), the P- and R- variants of *P. aurantiaca* had the same growth characteristics as the dominant S-type. However, when grown in a medium with a twofold nitrogen limitation, these variants exhibited considerable differences (Fig. 3a). Thus, the pigmented P-variant grew at the highest rate (μ_{max} = 0.046 h^{-1}), had the maximum biomass yield (Y_{max} = 1 g DCM/l), and was best adapted to nitrogen deficiency. The R-variant, while growing on nitrogen-limited medium, showed low μ_{max} and Y_{max} (0.035 h^{-1} and 0.7 g DCM/l, respectively), whereas the dominant S-variant had the lowest growth rate under these conditions (Table 2). Thus, the differences between the P-, R-, and S-variants in their adaptation to new environmental conditions were determined by the medium quality, i.e., the content of nutrients (50% nutrient broth) or the deficiency in one of the nutrient compounds (nitrogen).

Another demonstrated difference between the S-, P- and R-type variants of *P. aurantiaca* concerned their ability to grow in the presence of streptomycin and tetracycline in 50% nutrient broth, the medium, in which the variants exhibited the same growth characteristics. Based on the dependence of growth inhibition on antibiotic concentrations, LD_{50} and LD_{70} values were determined. Analysis of these parameters did not reveal any significant differences between the variants in their antibiotic sensitivity. P- and R-variants were more sensitive to streptomycin (LD_{50} = 155 and 180 μg/ml, respectively) than the dominant S-variant (LD_{50} = 235 μg/ml). The S-type was more sensitive to tetracycline (LD_{50} = 25 μg/ml) then the P- and R-variants (LD_{50} = 65 and 40 μg/ml, respectively) (Table 2). In addition, the *P. aurantiaca* variants differed in their extracellular protease activity; in the cultures grown in 50% nutrient broth, the dominant S-type and the R-variant exhibited the highest and the lowest protease activity respectively, at the onset of the stationary growth phase (Table 2).

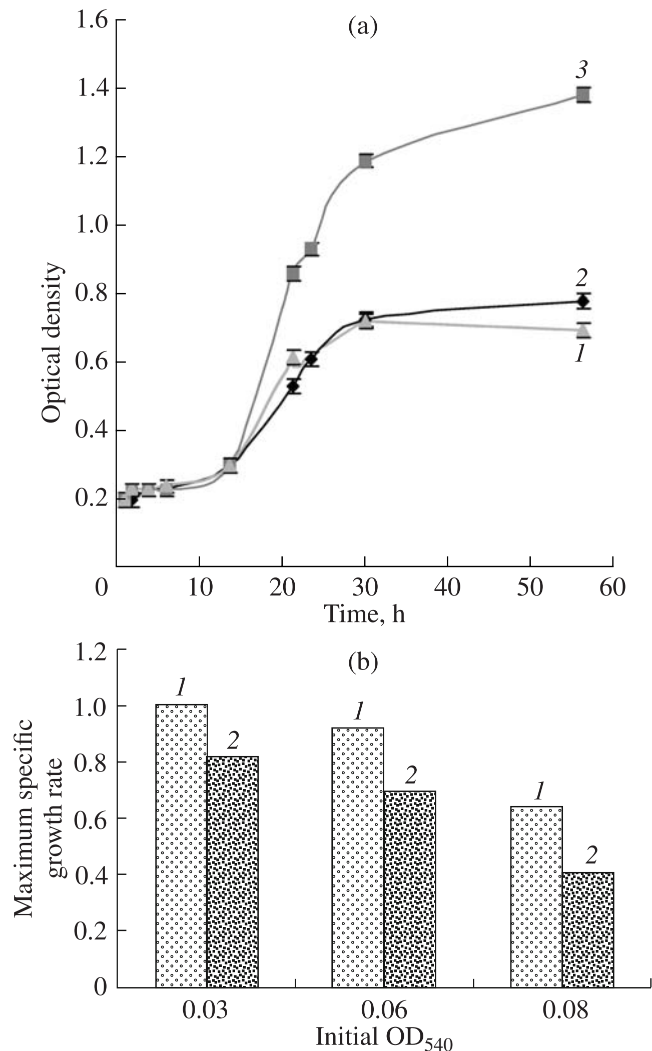

Fig. 3. Growth of *P. aurantiaca* variants in synthetic nitrogen-limited medium (a) and dependence of the specific growth rate of *P. fluorescens* variants on the inoculum size (initial OD) (b). Designations: *1*, S-type; *2*, R-type; and *3*, P-type of *P. aurantiaca*.

Analysis of the growth characteristics of R- and S-types of *P. fluorescens* did not reveal any significant differences (Table 3). Thus, the rate (μ_{max}) of the R-type growth on balanced synthetic medium M9 was 20%

Table 2. Differences between *P. aurantiaca* variants in physiological and biochemical properties

Types of *P. aurantiaca* variants	Growth characteristics*		Resistance (LD_{50}, μg/ml) to		Extracellular protease activity, mE/ml
	Biomass yield (Y_{max}), g DCM/l	Maximum growth rate (μ_{max}), h^{-1}	streptomycin	tetracycline	
R	0.7	0.035	180	40	5.3
S	0.65	0.024	235	25	14.8
P	1	0.046	155	65	13

* Nitrogen-limited medium. Deviation values ±5%.

Table 3. Correlation between sensitivity to the chemical analogues of microbial anabiosis autoinducers (C_6-AHB and C_{12}-AHB), enhanced stress resistance, and the growth characteristics of *P. fluorescens* variants

Types of *P. fluorescens* variants	Growth characteristics*		Sensitivity to d_1 factor analogues		Resistance (LD_{50}), μg/ml to	
	Y_{max}, g DCM/l	μ_{max}, h^{-1}	C_7-AHB	C_{12}-AHB	NaCl	H_2O_2
R	0.50	0.4055	Growth stimulation: 50–200 μM; Growth inhibition: 300–700 μM; Growth inhibition by 50%: 1220 μM	Growth stimulation: 5–15 μM; Growth inhibition: >30–50 μM; Growth inhibition by 50%: 54 μM	380 mM	1800 μM
S	0.64	0.6419	No effects on growth at a concentration of 50–1000 μM; Growth inhibition by 50%: 2600 μM	Growth stimulation: 70 μM; Growth inhibition >150–400 μM; Growth inhibition by 50%: 370 μM	185 mM	600 μM

* Growth on M9 medium with an initial OD value of 0.08. Deviation values ±5%.

lower than that of the dominant S-variant; the biomass yield was lower as well. The differences in μ_{max} depended on the initial size of inoculum (0.03–0.08) and became more pronounced with the increase of its amount (Fig. 3b). The low growth rate of the R-variant can be due to enhanced production of extracellular and intracellular metabolites, having growth-inhibiting effects, as in the case of *P. aeruginosa* [13]. Accordingly, the content of inhibitors increased with an increase in the amount of inoculum. This function is inherent to microbial anabiosis autoinducers, represented in some pseudomonads by alkylhydroxybenzene (AHB) isomers and homologues [4, 6]. Differences between R- and S-variants of *P. fluorescens* in the AHB production can be assessed indirectly from the sensitivity to an increase of the extracellular level of these metabolites due to their exogenous introduction. Therefore, in subsequent experiments, we assessed the effect of two analogues of microbial AHB, C_7-AHB and C_{12}-AHB with different alkyl chain length, added in concentrations of 5 μM–2 mM, on the growth of studied variants in comparisons with control cultures (without AHB). It was demonstrated that the addition of C_7-AHB (50–200 μM) stimulated the growth of *P. fluorescens* R-variant by 10–20%, whereas the addition of 300–700 μM C_7-AHB inhibited its growth by 10–30%. C_7-AHB had no noticeable effect on the growth of the S-variant (Fig. 4a). However, the responses of R- and S-variants to addition of C_{12}-AHB differed significantly. Thus, C_{12}-AHB exerted a growth-stimulating effect on the R-type in a narrow concentration range (5−15 μM) and inhibited its growth by 15–50% at concentrations of 30–50 μM, in contrast to the S-type, whose growth was inhibited by C_{12}-AHB concentrations in a range of 150–500 μM (Fig. 4b). Hence, the R-variant of *P. fluorescens* was more sensitive to high concentrations of exogenously added AHB than the wild type S-variant. These results suggest the presence of high levels of intrinsic intracellular AHB, bacterial growth autoregulators, which is in accordance with the data on high productivity of *P. aeruginosa* R-variant in alkylhydroxybenzenes [13].

Being the cause of its relatively slow growth, an elevated AHB level in the culture of *P. fluorescens* R-variant may also contribute to enhanced resistance of its cells to unfavorable conditions and damaging factors, as proved by our experiments with creation of different stressful factors. We found that R-variant was generally more tolerant to osmotic stress (high salinity), than S-variant as judged from the revealed dependence of their growth on NaCl concentrations in the medium

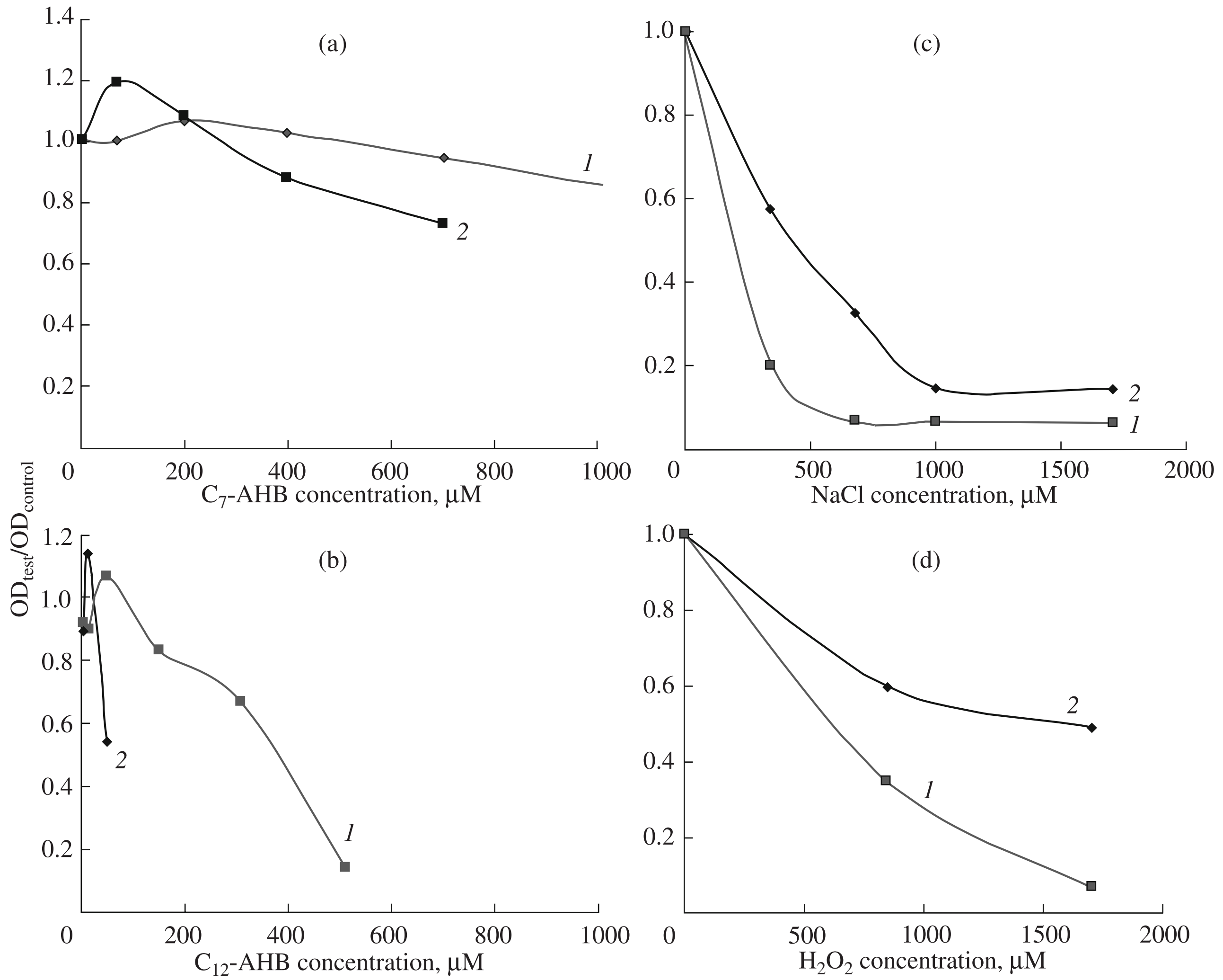

Fig. 4. Influence of (a) C_7-AHB, (b) C_{12}-AHB, (c) NaCl, and (d) H_2O_2 on the growth of (*1*) S- and (*2*) R-variants of *P. fluorescens*.

(Fig. 4c) and LD_{50} values (Table 3). Also, R-type was more resistant to H_2O_2-induced (170–2000 μM) oxidative stress than S-variant (Fig. 4d), as seen from the corresponding LD_{50} values (1800 and 600 μM, respectively; Table 3). Thus, comparative analysis of the R- and S-variants of *P. fluorescens* indicate a correlation between their growth characteristics, resistance to various stress factors, and differences in the production of autoregulators for growth and development of microbial cultures, which was confirmed indirectly in our experiments by the reaction to exogenous AHB addition.

DISCUSSION

The major result of this work was the demonstration of another level in the biodiversity of dormant bacterial forms. This level manifests itself in the different ability of *P. aurantiaca* and *P. fluorescens* CRC to give rise to phenotype variants. Intrapopulational variability depended on the conditions of CRC formation and storage time (age of resting cells). An increase in the variability index, as judged by appearance of the variants with different colony morphology and physiological and biochemical characteristics was also demonstrated for the germination of *B. cereus* CRC obtained under various conditions of spore formation repression [9], as well as for anabiotic cells of *S. aureus* [11], *Salmonella typhimurium* [14], and *P. aurantiaca* [12] whose formation was induced by AHB. Hence, the results of this and cited works suggest that increased phenotypic variability can be regarded as a salient feature of bacterial cyst-like resting forms. Since cyst-like cells are widespread in natural habitats [4, 5], their high phenotypic variability constitutes an adaptive mechanism for microbial populations and promotes survival of bacterial species.

A relationship between the dormant state and the phenotypic variability can be suggested from the studies of *P. aeruginosa* biofilms [15–19]. Generally, the development of these bacteria in biofilms favored the emergence of (1) small S-type colonies with reduced

motility and enhanced adherence (strain *P. aeruginosa* 57RP) [15]; (2) mini-colonies and rough colonies differing in the ability to produce biofilms (various *P. aeruginosa* strains) [16]; and (3) small-type (ST) wrinkled colonies (*P. aeruginosa* PAO1) [17], all differing from the wild type colonies. According to the conception [18], biofilms contain “absolutely dormant” cells; however, these cells were assigned to this category solely because the majority of the biofilm population did not exhibit alkaline phosphatase activity [19].

The formation of RSCV (rough small-colony) variants with a high resistance to a wide range of antibiotics was observed upon plating of *P. aeruginosa* onto solid media supplemented with kanamycin [20]. Emergence of *P. aeruginosa* RSCV is probably associated with the transition of a part of the microbial population to the dormant state that is also characterized by resistance to antimicrobial agents. It is noteworthy that antibiotic-resistant, persistor cells of *E. coli* differ from actively growing and stationary phase cells in the expression of several genes and in the transcriptome profile and exist in a specific physiological state associated with proliferative dormancy [21].

Finally, F- and S-variants of *P. fluorescens* strain F113, differing in their colony morphology, motility, and productivity of some bioactive compounds, were isolated from the alfalfa rhizosphere inoculated with the wild-type variant and incubated for four weeks [22]. On the basis of population analysis of the marker *P. fluorescens* strain introduced into the rhizosphere, it was concluded that some cells were in a dormant state and showed heterogeneity in their ability to produce colonies and microcolonies [23].

Our studies with *P. aurantiaca* and *P. fluorescens* provided direct evidence for the relationship between phenotypic variability and dormancy, as well as its dependence on the properties of CRC and conditions of their formation, viz. cultivation in nitrogen-limited media, the influence of elevated concentrations of anabiosis autoinducers (C_{12}-AHB analogues), and during prolonged storage time (Table 1, Fig. 1). Based on the data obtained, we may explain the enhanced phase variation of pseudomonads occurring in the rhizosphere and biofilms or developing under the influence of antibiotics. It is important to note the influence of solid medium composition on the development of the *P. fluorescens* variants that appeared after CRC plating onto broth-wort agar, but not on nutrient agar (Fig. 2). It is well known that the development of colony morphology variants depends upon the composition of the growth media. In particular, the emergence frequency of *P. aeruginosa* RSCV was high when modifications were made to the solid nutrient media (LB, LB with NaCl, and minimal medium), as well as to the incubation temperature (25°C and 37°C) [20].

Phenotypic (phase) variability in bacteria is caused by reversible genomic rearrangements. In the representatives of the genus *Pseudomonas*, the phenotypic variability is due to several mechanisms, namely: (1) frameshift mutations in the poly(G) fragment of the *flhB* gene of *P. putida* DOT-T1E (phase variation of swimming) [24]; (2) a 661-bp spontaneous, reversible duplication in the regulatory *pheN* gene of *P. tolaasii* (transition from the S variant, which is pathogenic to fungi, to the nonpathogenic R-variant) [25]; (3) expression of site-specific recombinase encoded by the *sss* gene in *P. fluorescens* F113 (emergence of the F and S variants) [22]; and (4) accumulation of spontaneous reversible mutations in the regulatory *gacA*/*gacS* genes in *Pseudomonas* sp. (strain PCL 1171) (phase variation from type I opaque colonies to type II translucent colonies) [26], which is positively regulated by the *rpoS* gene (a global regulator of genes expressed in stationary-phase cells), especially when the mismatch repair system encoded by the *mutS* gene is not efficient [27]. It should be emphasized that phenotypic variability is associated with events occurring during the stationary phase and in particular with mutations [28].

Evidently, genetic mechanisms and events responsible for phenotypic variation in pseudomonad species are different. The species-non-specific regulation of phenotypic variability is notable in this connection; it is based on the effect of low-molecular-weight metabolites, alkylhydroxybenzenes (AHB), with functions of anabiosis autoinducers. Results of our previous studies, as well as the data obtained during the above-described experiments with two *Pseudomonas* species demonstrate that certain AHB homologues (C_{12}-AHB) induce phase transitions in gram-positive and gram-negative bacteria [9–12, 14]. The mechanism involved in the regulation of phenotypic variation by AHB may be associated with the ability of AHB to act as chemical chaperones and affect the conformation and activity of proteins responsible for gene expression at the transcriptional level [4], as well as with the direct interaction with DNA of various origin [12, 29, 30]. The latter hypothesis seems to be more plausible since it was confirmed by the following facts: (1) weak mutagenic activity of some AHB homologues (C_{12}-AHB) revealed by the Ames test with the *S. typhimurium* auxotrophic trp mutant (frameshift mutation) [14]; (2) capability of AHB to induce an SOS-response [31]; and (3) accumulation of anabiosis autoinducers (AHB of the alkylresorcinol group) in the stationary-phase and dormant cells of *Pseudomonas* species (and other microorganisms) [4, 6, 7]. It was shown that interaction of AHB with individual DNA, resulting in changes in its physicochemical properties and topology (B $\longrightarrow$ A transition), developed as time passed [30]. This may explain the dependence of phenotypic variability on storage time (20 days–4 months) of *P. aurantiaca* and *P. fluorescens* CRC (Table 1, Fig. 1). This dependence was also confirmed by the data on changes in the spectrum of mutations associated with phenotypic variability during ageing of starved *P. putida* cultures [32].

The differences of the variants developing from *P. fluorescens* and *P. aurantiaca* CRC of various types, in growth characteristics, as well as resistance to antibiotics, high salinity, and oxidative stress (Figs. 3 and 4; Tables 2 and 3) reflect their different abilities to adapt to changing environmental conditions, which agrees with the published data. For instance, enhanced resistance of the *P. fluorescens* R-variant to H_2O_2 (as compared to the S-type (Fig. 4)) was also shown for a similar colony morphology *P. aeruginosa* variant growing in biofilms, but not in a planktonic culture [16]. The actively growing cells of *P. aeruginosa* 57RP S-variant, producing small colonies, showed higher sensitivity to H_2O_2 than the initial L-type [15]. RSCV and the wild type of *P. aeruginosa* differed in their resistance to kanamycin [20].

The ability of *P. aurantiaca* and *P. fluorescens* to produce CRC of various morphotypes that exhibit different survivability, thermal resistance [1], and capacity for phenotypic radiation, resulting in the emergence of colony morphology variants with adaptability to new (stress) conditions provide further insight into the survival mechanisms of these non-spore-forming bacteria.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project nos. 07-04-01011 and ofi_07-04-12121).

REFERENCES

1. Mulyukin, A.L., Suzina, N.E., Duda, V.I., and El'-Registan G.I. Structural and Physiological Diversity among Cystlike Resting Cells of Bacteria of the Genus *Pseudomonas, Mikrobiologiya*, 2008, vol. 77, no. 4, pp. 512–523 [*Microbiology* (Engl. Transl.), vol. 77, no. 4, pp. 455–465].
2. Mulyukin, A.L., Lusta, K.A., Gryaznova, M.N., Kozlova, A.N., Duzha, M.V., Duda, V.I., and El'-Registan, G.I., Formation of Resting Cells by *Bacillus cereus* and *Micrococcus luteus, Mikrobiologiya*, 1996, vol. 65, no. 6, pp. 782–789 [*Microbiology* (Engl. Transl.), vol. 65, no. 6, pp. 683–689].
3. Demkina, E.V., Soina, V.S., and El'-Registan, G.I., Formation of Resting Forms of *Arthrobacter globiformis* in Autolyzing Cell Suspensions, *Mikrobiologiya*, 2000, vol. 69, no. 3, pp. 383–388 [*Microbiology* (Engl. Transl.), vol. 69, no. 3, pp. 314–318].
4. El'-Registan, G.I., Mulyukin, A.L., Nikolaev, Yu.A., Suzina, N.E., Gal'chenko, V.F., and Duda, V.I., Adaptogenic Functions of Extracellular Autoregulators of Microorganisms, *Mikrobiologiya*, 2006, vol. 75, no. 4, pp. 446–456 [*Microbiology* (Engl. Transl.), vol. 75, no. 4, pp. 380–389].
5. Suzina, N.E., Mulyukin, A.L., Kozlova, A.N., Shorokhova, A.P., Dmitriev, V.V., Barinova, E.S., Mokhova, O.N., El'-Registan, G.I., and Duda, V.I., Ultrastructure of Resting Cells of Some Non-Spore-Forming Bacteria, *Mikrobiologiya*, 2004, vol. 73, no. 4, pp. 516–529 [*Microbiology* (Engl. Transl.), vol. 73, no. 4, pp. 435–447].
6. Svetlichnyi, V.A., Romanova, A.K., and El'-Registan, G.I., Qualitative Content of Membrane-Active Autoregulators under Lithoautotrophic Growth, *Mikrobiologiya*, 1986, vol. 55, no. 1, pp. 55–59.
7. Osipov, G.A., El'-Registan, G.I., Svetlichnyi, V.A., Kozlova, A.N., Duda, V.I., Kaprel'yants, A.S., and Pomazanov, V.V., On the Chemical Nature of the *Pseudomonas carboxydoflava* d Autoregulatory Factor, *Mikrobiologiya*, 1985, vol. 54, no. 2, pp. 186–190.
8. Mulyukin, A.L., Kozlova, A.N., Kaprel'yants, A.S., and El'-Registan, G.I., The d_1 Autoregulatory Factor in *Micrococcus luteus Cells and Culture Liquid: Detection* and Accumulation Dynamics, *Mikrobiologiya*, 1996, vol. 65, no. 1, pp. 20–25 [*Microbiology* (Engl. Transl.), vol. 65, no. 1, pp. 15–20].
9. Doroshenko, E.V., Loiko, N.G., Il'inskaya, O.N., Kolpakov, A.I., Gornova, I.V., and El'-Registan, G.I., Characterization of *Bacillus cereus* Dissociants, *Mikrobiologiya,* 2001, vol. 70, no. 6, pp. 811–819 [*Microbiology* (Engl. Transl.), vol. 70, no. 6, pp. 698–705].
10. Khabibullin, S.S., Nikolaev, Yu.A., Loiko, N.G., Golod, N.A., Mil'ko, E.S., Voeikova, T.A., and El'-Registan, G.I., Autoregulation of Phenotypic Dissociation in *Bacillus licheniformis, Zhur. Mikrobiol., Epidemiol. Immunobiol.,* 2006, no. 6, pp. 9–13.
11. Il'inskaya, O.N., Kolpakov, A.I., Shmidt, M.A., Doroshenko, E.V., Mulyukin, A.L., and El'-Registan, G.I., The Role of Bacterial Growth Autoregulators (Alkyl Hydroxybenzenes) in the Response of Staphylococci to Stresses, *Mikrobiologiya,* 2002, vol. 71, no. 1, pp. 23–29 [*Microbiology* (Engl. Transl.), vol. 71, no. 1, pp. 18–23].
12. Mulyukin, A.L., Vakhrushev, M.A., Strazhevskaya, N.B., Shmyrina, A.S., Zhdanov, R.I., Suzina, N.E., Duda, V.I., Kozlova, A.N., and El'-Registan, G.I., Effect of Alkylhydroxybenzenes, Microbial Anabiosis Inducers, on the Structural Organization of *Pseudomonas aurantiaca* DNA and on the Induction of Phenotypic Dissociation, *Mikrobiologiya*, 2005, vol. 74, no. 2, pp. 157–165 [*Microbiology* (Engl. Transl.), vol. 74, no. 2, pp. 128–135].
13. Mil'ko, E.S., Khabibullin, S.S., Nikolaev, Yu.A., Kozlova, A.N., and El'-Registan, G.I., Dynamics of the Growth and Population Composition of Mixed Cultures of R, S, and M Dissociants of *Pseudomonas aeruginosa,* Mikrobiologiya, 2005, vol. 74, no. 4, pp. 475–482 [*Microbiology* (Engl. Transl.), vol. 4, no. 4, pp. 408–414].
14. Il'inskaya, O.N., Kolpakov, A.I., Zelenin, P.V., Kruglova, Z.D., Choidash, B., Doroshenko, E.V., Mulyukin, A.L., and El'-Registan, G.I., The Effect of Anabiosis Autoinducers on the Bacterial Genome, *Mikrobiologiya*, 2002, vol. 71, no. 2, pp. 194–199 [*Microbiology* (Engl. Transl.), vol. 71, no. 2, pp. 164–168].
15. Déziel, E., Comeau, Y., and Villemur, R., Initiation of Biofilm Formation by *Pseudomonas aeruginosa* 57RP Correlates with Emergence of Hyperpiliated and Highly Adherent Phenotypic Variants Deficient in Swimming, Swarming, and Twitching Motilities, *J. Bacteriol.*, 2001, vol. 183, pp. 1195–1204.
16. Boles, B.R., Thoendel, M., and Singh, P.K., Self-Generated Diversity Produces 'Insurance Effects' in Biofilm

Communities, *Proc. Natl. Acad. Sci. USA*, 2004, vol. 101, no. 47, pp. 16630–16635.

17. Kirisits, M.J., Prost, L., Starkey, M., and Parsek, M.R., Characterization of Colony Morphology Variants Isolated from *Pseudomonas aeruginosa* Biofilms, *Appl. Environ. Microbiol.*, 2005, vol. 71, pp. 4809–4821.
18. Costerton, J.W., Camper, A.K., Stewart, P.S., Zelver, N., and Dirckx, M.E., The Problem: Not Just Bacteria-Bacterial Biofilms, *The Analyst*, 1999, vol. 6, no. 3, pp. 18–25.
19. Xu, K.D., Stewart, P.S., Xia, F., Huang, C.-T., and McFeters, G.A., Spatial Physiological Heterogeneity in *Pseudomonas aeruginosa* Biofilm Is Determined by Oxygen Availability, *Appl. Environ. Microbiol.,* 1998, vol. 64, no. 10, pp. 4035–4039.
20. Drenkard, E. and Ausubel, F.M., Pseudomonas Biofilm Formation and Antibiotic Resistance Are Linked to Phenotypic Variation, *Nature,* 2002, vol. 416, pp. 740–743.
21. Shah, D.V., Zhang, Z., Kurg, K., Kaldalu, N., Khodursky, A., and Lewis, K., Persisters: a Distinct Physiological State of *Escherichia coli, BMC Microbiol.*, 2006, vol. 6, p. 53.
22. Sanchez-Contreras, M., Martin, M., Villacieros, M., O'Gara, F., Bonilla, I., and Rivilla, R., Phenotypic Selection and Phase Variation Occur During Alfalfa Root Colonization by *Pseudomonas fluorescens* F113, *J. Bacteriol.*, 2002, vol. 184, pp. 1587–1596.
23. Normander, B., Hendriksen, N.B., and Nybroe, O., Green Fluorescent Protein-Marked *Pseudomonas fluorescens*: Localization, Viability, and Activity in the Natural Barley Rhizosphere, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 4646–4651.
24. Segura, A., Hurtado, A., Duque, E., and Ramos, J.L., Transcriptional Phase Variation at the *flhB* Gene of *Pseudomonas putida* DOT-T1E Is Involved in Response to Environmental Changes and Suggests the Participation of the Flagellar Export System in Solvent Tolerance, *J. Bacteriol.,* 2004, vol. 186, pp. 1905–1909.
25. Han, B., Pain, A., and Johnstone, K., Spontaneous Duplication of a 661 bp Element within a Two-Component Sensor Regulator Gene Causes Phenotypic Switching in Colonies of *Pseudomonas tolaasii*, Cause of Brown Blotch Disease of Mushrooms, *Mol. Microbiol.*, 1997, vol. 25, pp. 211–218.
26. van den Broek, D., Chin-A-Woeng, T.F.C., Bloemberg, G.V., and Lugtenberg, B.J.J., Molecular Nature of Spontaneous Modifications in *gacS* Which Cause Colony Phase Variation in *Pseudomonas* sp. PCL1171, *J. Bacteriol.*, 2005, vol. 187, pp. 593–600.
27. van den Broek, D., Chin-A-Woeng, T.F.C., Bloemberg, G.V., and Lugtenberg, B.J.J. Role of RpoS and MutS in Phase Variation of *Pseudomonas* sp. PCL1171, *Microbiology. (UK)*, 2005, vol. 151, pp. 1403–1408.
28. Kivisaar, M., Stationary Phase Mutagenesis: Mechanisms That Accelerate Adaptation of Microbial Populations under Environmental Stress, *Environ. Microbiol.*, 2003, vol. 5, pp. 814–827.
29. Davydova, O.K., Deryabin, D.G., Nikiyan, A.N., and El'-Registan, G.I., Mechanisms of Interaction between DNA and Chemical Analogues of Microbial Anabiosis Autoinducers, *Mikrobiologiya*, 2005, vol. 74, no. 5, pp. 616–625 [*Microbiology* (Engl. Transl.), vol. 74, no. 5, pp. 533–541].
30. Davydova, O.K., Deryabin, D.G., and El'-Registan, G.I., IR Spectroscopic Research on the Impact of Chemical Analogues of Autoregulatory d_1 Factors of Microorganisms on Structural Changes in DNA, *Mikrobiologiya*, 2007, vol. 76, no. 3, pp. 306–312 [*Microbiology* (Engl. Transl.), vol. 76, no. 3, pp. 266–272].
31. Margulis, A.B., Il'inskaya, O.N., Kolpakov, A.I., and El'-Registan, G.I., Induction of SOS Response by Autoregulatory Factors of Microorganisms, *Genetika,* 2003, vol. 39, no. 9, pp. 1180–1186 [*Russ. J. Genet.* (Engl. Transl.), vol. 39, no. 9, pp. 993–996].
32. Saumaa, S., Tover, A., Kasak, L., and Kivisaar, M., Different Spectra of Stationary-Phase Mutations in Early-Arising Versus Late-Arising Mutants of *Pseudomonas putida*: Involvement of the DNA Repair Enzyme MutY and the Stationary-Phase Sigma Factor RpoS, *J. Bacteriol.*, 2002, vol. 184, pp. 6957–6965.