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EXPERIMENTAL ARTICLES

Effect of a Dormant State on the Xenobiotic-Degrading Strain *Pseudomonas fluorescens* 26K

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Abstract—The changes in physiological and biochemical properties of *Pseudomonas fluorescens* 26K, a degrader of chlorinated aromatic compounds, were revealed after existing in a dormant state as cyst-like cells (CLC). The CLC maintained the ability to form colonies after long-term storage, possessed enhanced resistance to damaging factors (heating and drying), and had specific ultrastructural organization. In populations grown from CLC on solid media, we observed the appearance of phenotypic variants, which differed from the dominant type in the shape, consistency, and pigmentation of colonies. The emerging phenotypes had higher growth rates on some aromatic substrates, which required the enzymes with broadened substrate specificity for their utilization.

Keywords: Pseudomonas, dormant forms, resistance, xenobiotic biodegradation, enzymatic activity

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Many species of bacteria of the genus *Pseudomonas* are capable of degrading a variety of xenobiotics such as chlorobenzoates, chlorophenols, chlorinated biphenyls, and their unsubstituted analogs. The ability of pseudomonads to carry out the degradation reactions is due to a set of enzymes with different substrate specificity. Pseudomonads are characterized by high metabolic activity, high growth rate, and multiple isoenzymes, which makes them promising for bioremediation of contaminated ecotopes [1–5]. However, under natural conditions, the period of metabolic activity of a given population of pseudomonads is rather short, followed by transition to the dormant state, thus giving way to the population of a different biodegrading strain. Therefore, it is expedient to use active associations instead of individual degrading bacteria. Good results were obtained when associations of bacteria belonging to different taxonomic groups and complementing each other by their physiological-biochemical properties were created. An example of such an approach is the use of bacteria of two genera, *Pseudomonas* and *Rhodococcus*, for the development of binary bacterial preparations for bioremediation of oil-contaminated soils and degradation of waste gases [4–7].

Earlier, we showed that bacteria of the genus *Rhodococcus*, as other non-spore-formers, are capable of forming cystlike dormant cells (CLC) intended for long-term survival under no-growth or unfavorable conditions. When the CLC of *Rhodococcus opacus* 1cp germinated, phenotypic variants possessing a substantially enhanced biodegrading potential developed [8]. Apparently, this was caused by the emerging phenotypes being able to realize the necessary biodegrading pathway, which significantly shortened the time of culture adaptation to new substrates. As for pseudomonads, it is known that under laboratory conditions and in soil microcosms they are not tolerant of starvation and lose the capacity for colony formation [9, 10]. Therefore, it was of interest both to reveal the cell forms of pseudomonad survival under no-growth conditions and to determine the characteristics of the phenotypes grown after inoculation of fresh media with dormant forms.

The goal of this work was to obtain the dormant forms of *Pseudomonas fluorescens* 26K, to study their morphology and ultrastructural organization, as well as to investigate the degrading potential of the pseudomonad cultures developing from the dormant cells.

MATERIALS AND METHODS

The microorganism and the cultivation conditions. The gram-negative non-spore-forming bacterium *Pseudomonas fluorescens* 26K isolated from the waste waters of a coal-tar chemical production of the Karaganda metallurgic plant and capable of growing

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on naphthalene, *m*- and *p*-cresol, phenanthrene, salicylic and phthalic acids, 3,4-dichloro- and 3,4-difluoroaniline [11, 12], and transforming fluorene [13], was the subject of study.

The strain was maintained by transfers on LB agar slants. In order to obtain the inoculum, *P. fluorescens* cultures were grown on a rich medium—LB broth at 28°C in 250-mL flasks with 50 mL of medium on a shaker (220 rpm) up to the exponential growth phase.

Assays of cell resistance to antibiotics. The resistance of the strain to antibiotics was assessed by the delay in, or the absence of, cell growth in the liquid medium LB (in 48-well plates) into which kanamycin, tetracycline, or ampicillin (0.01–200 μg/mL) were added together with the inoculum (10^7 cells/mL). The plates were incubated at 28°C under static conditions for 24–72 h and periodically examined to reveal the antibiotic concentrations determining the bactericidal effect.

Obtaining of dormant forms. The cystlike dormant cells (CLC) were obtained in aging cultures developing (1) in a depleted nutrient medium with a two- and fivefold decreased N source concentration, and (2) upon addition of the chemical analogue of microbial anabiosis autoinducers C_{12}-AHB (M = 194) at concentrations of 1×10^{-4}–7×10^{-4} M into the stationary-phase pseudomonad cultures grown in LB broth, and (3) in the cultures growing on soil agar in petri dishes with subsequent transfer with 5 mL of saline solution from each dish. A detailed description of the protocols used to obtain CLC is given in [14].

Total cell number in the suspensions was assessed by direct microscopy counting in 20 small squares (25 μm^2) in a Goryaev chamber. The colony-forming capacity of the cells (CFU/mL) was assessed by plating tenfold dilutions of the suspensions on LB agar (1.5% agar wt/vol) with incubation at 28°C for 72 h. The most probable number of viable cells (MPN/mL) was evaluated by the bacterial growth after serially diluted inoculations of LB broth in multiwell plates (50 μL of suspension in 450 μL of LB broth).

Thermal resistance was assessed as the number of the cells that remained viable after heating the suspensions (0.7 mL) in a thermostat at 50, 55, and 60°C for 5 min.

Microscopy examinations were performed using a Zetopan light microscope (Reichert, Austria) and an Axioplan microscope equipped with a phase contrast device (Carl Zeiss, Germany). For electron microscopic studies, the precipitated cells were fixed in 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed off thrice in the same buffer, and additionally fixed in 1% OsO_4 solution in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20°C. After dehydration, the material was embedded in the Epon 812 epoxide resin. Ultrathin sections were contrasted for 30 min with 3% uranyl acetate solution in 70% alcohol and additionally stained with lead citrate according to Reynolds [15] at 20°C for 4–5 min. The sections were examined under a JEM-100B electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

The phenotypic variability of the strain *P. fluorescens* 26K was estimated by the emergence of variants differing from the dominant phenotype in the colony shape, consistency, and pigmentation. The dominant S-variant was the initial phenotype used to obtain CLC and investigating the phenotypic variability. Solid media with kanamycin (10 μg/mL) were used to reveal the antibiotic-resistant variants. The phenotypic variability index was determined as a percentage of certain types of colonies in relation to the total number of colonies. The multiple passage method was used to study the stability of the variants.

Assessment of the utilization of aromatic substrates. The rich LB agar medium was inoculated with CLC suspensions (10–15 μL). The adaptation of *P. fluorescens* 26K cultures grown upon inoculation of CLC or vegetative cells to toxic substrates was carried out by multiple transfers on the mineral agar medium containing phenol; 2-chlorophenol (2-CP); 2,3-; 2,4-; 2,5-; 2,6-; 3,4-dichlorophenols (DCP); 2,3,4-; 2,4,5-; 2,4,6-trichlorophenols (TCP); pentachlorophenol (PCP); 2-, 3-, 4-chlorobenzoates (CB); 2,4-; 2,5-; 2,6-; 3,5-dichlorobenzoates (DCB); *p*-hydroxybenzoate (PHB); gentisate; benzoate; salicylate; 5-chlorosalicylate; and *p*-toluate. Cell viability after four sequential transfers was assessed by the presence of growth when the cells were inoculated into test tubes with tenfold diluted LB-broth (7–10 mL) and cultivated in a shaker (29°C, 220 rpm) for five days.

In order to study the growth characteristics of *P. fluorescens* 26K on the aromatic substrates, a liquid mineral medium was used containing the following (g/L): Na_2HPO_4, 0.73; KH_2PO_4, 0.35; $MgSO_4 \cdot 7H_2O$, 0.1; $NaHCO_3$, 0.25; $MnSO_4$, 0.002; NH_4NO_3, 0.75; $FeSO_4 \cdot 7H_2O$, 0.02. The medium was supplemented with specific growth substrates as the only source of carbon and energy. A trace element solution (1 mL/L) of the following composition (mg/L) was added to the medium: $ZnSO_4$, 0.30; $CoCl_2 \cdot 6H_2O$, 0.70; $(NH_4)_6Mo_7O_{24}$, 0.86; $CuSO_4 \cdot 5H_2O$, 0.25. The biomass grown on agar was washed off with the mineral medium and cultivated in 750-mL Erlenmeyer flasks containing 100 mL of the medium (29°C, 220 rpm). The sole carbon and energy sources used were *p*-toluate (70 mg/L), 2,6-DCB (50 mg/L), 3-CB (200 mg/L), PHB (70 mg/L), gentisate (400 mg/L), and sodium benzoate (200 mg/L).

The growth characteristics of cultures—the maximum specific growth rate (μ) and the doubling time (t_d)—were calculated based on the results of measurement of the suspension optical density (λ = 545 nm, l = 10 mm, using a Shimadzu UV-160 spectrophotometer, Japan). The residual concentration of the

substrate was determined by the UV spectra of the supernatant at $\lambda = 200–400$ nm.

Assays of enzymatic activities in cell-free extracts. The bacteria were cultivated in 750-mL flasks (28°C, 220 rpm) containing 200 mL of the medium with 3-CB (200 mg/L). The cultures were grown up to the optical density 1.8 (upon inoculation with the cultures grown from the dormant forms) and 1.6 (upon inoculation with the stationary phase culture grown in LB broth). The cells were separated by centrifugation (12000 *g*, 10 min, Beckmann J2-21 centrifuge), washed off twice with 50 mM Tris-HCl buffer (pH 7.2) with centrifugation (16000 *g*, 15 min), and frozen. In order to obtain the biomass on gentisate and sodium benzoate, the cultures were grown to $OD_{545} = 1–1.5$, the cells were collected and stored in a similar way. The biomass was degraded by extrusion disintegration on a Hughes-type press with a working pressure of 3200 kg/cm^2, incubated with DNase (15 min, 20°C), and centrifuged (30000 *g*, 30 min, 5°C). The sediment was resuspended in 50 mM Tris-HCl buffer (pH 7.2) with 2 mM $MnSO_4$ and centrifuged under the same conditions. The supernatant fluids obtained were pooled and centrifuged in the same mode to obtain a transparent cell-free extract.

The enzyme activity was determined at 25°C spectrophotometrically (quartz cells, $l = 10$ mm, a Shimadzu UV-160 spectrophotometer, Japan). The activity of catechol-1,2-dioxygenase, methylcatechol 1,2-dioxygenase, and chlorocatechol 1,2-dioxygenase was determined using the modified method [16]. The reaction was started by addition of the enzyme; its activity was calculated by the product formation rate at $\lambda = 260$ nm using the molar extinction coefficients [17]. The amount of the enzyme catalyzing the conversion of 1 μmol of a substrate or the formation of 1 μmol of a product per minute was accepted as a unit activity. The relative activity was calculated taking an activity with an unsubstituted or a better substrate to be 100%.

The protein concentration was determined with the modified method of Bradford [18] using bovine serum albumin as the standard.

Two independent series of the experiments were staged with three replicates of measurements. These results reflect the averaged values. The statistical data analysis was carried out using Student's *t*-test taking $p < 0.05$ to be sufficient.

RESULTS AND DISCUSSION

Conditions for formation of the dormant pseudomonad cells. When the strain *P. fluorescens* 26 K was maintained on LB agar, the necessity for monthly transfers and the loss of capacity for quick restoration of growth on this medium after storage for two or three months were noted. Therefore, it seemed expedient to determine the conditions to induce formation of *P. fluorescens* 26K anabiotic cells capable of resuming the growth. The rich nutritive medium, LB broth, did not favor the formation of dormant forms: during long-term incubation of the cultures, the number of viable cells in the cultures decreased by 5 orders of magnitude and more (up to 10^4 CFU/mL after three months of storage). In order to intensify the formation of resting forms of the strain studied (the S-type dominant variant), the approaches developed for other species of pseudomonads [14], as well as for the strain *R. opacus* 1cp, a degrader of chlorine-containing xenobiotics, were applied [8].

Variant 1 of obtaining of dormant cells of *P. fluorescens* 26K was based on the use of the synthetic nutrient medium with 2- and 5-fold decreased concentrations of the nitrogen source. As was shown earlier, the C > N disbalance stimulates the biosynthesis of anabiosis autoinducers by bacteria [19]. The biomass yield, the efficiency of dormant cell formation by 26K, and the preservation of the colony-forming capacity depended on the degree of nitrogen limitation. Thus, the number of cells, which were grown in the medium with a twofold N limitation and maintained the ability to produce colonies, decreased over three months from 1×10^8 CFU/mL to $(3–5) \times 10^6$ CFU/mL, which constituted no more than 5% of the CFU titer in the stationary cultures. When nitrogen concentration in the medium was decreased fivefold, the total cell number and the CFU titer in the stationary pseudomonad cultures (5 days) were low (10^6 cells/mL and 1.5×10^4 CFU/mL, respectively). After two months of incubation, the CFU number decreased to zero values, although the MPN tests revealed a low (3×10^2 cells/mL) titer of the cells capable of resuming growth in LB broth. The dynamics of changes in the CFU number in stored *P. fluorescens* 26K cultures grown in N-limited media was similar to that shown by us for the other pseudomonad strains [14].

Thus, the C > N disbalance was not optimal for the formation of *P. fluorescens* 26K dormant cells with long-retained colony-forming capacity. This response of pseudomonads to conditions of strict nitrogen limitation in the growth medium differed from the behavior of the strain *R. opacus* 1cp, which formed cystlike dormant cells with a high degree of maintaining of the colony-forming capacity in the variant of development in the medium with a fivefold decrease in N source concentration [8].

Variant 2 to produce dormant (anabiotic) pseudomonad cells was based on the increase in the intracellular level of microbial anabiosis inducers by introducing their chemical analog C_{12}-AHB at a concentration of $5 \times 10^{-5}–1 \times 10^{-3}$ M into the early stationary-phase cultures grown in LB broth. The high number of anabiotic cells preserving the colony-forming capacity (30–45% of the initial CFU level) was noted in the variant with the introduction of C_{12}-AHB at concentrations of $1 \times 10^{-4}–1 \times 10^{-3}$ M. Although the

CFU titer in cultures of the strain 26K with an increased C_{12}-AHB concentration (7×10^{-4} M and 1×10^{-3} M) decreased in the dynamics of their storage (3–4 months), it was orders of magnitude higher than in the variants of cell growth in rich LB broth or under nitrogen limitation. The response to the increase in the C_{12}-AHB concentration to 1×10^{-3} M, at which the cells retained their colony-forming ability, was a feature of the strain *P. fluorescens* 26K. In other pseudomonad strains, (*P. aurantica* B-1558 and *P. fluorescens* NCIMB 904), lower C_{12}-AHB concentrations of $(2.5–5.0) \times 10^{-4}$ M caused a complete loss of the colony-forming ability [14].

Thus, the anabiotic dormant cells of *P. fluorescens* 26K formed when C_{12}-AHB was introduced into the cultures, and retaining the colony-forming capacity was of interest for searching the clones with improved biodegrading properties. It should be also noted that this variant of production of the dormant cells is promising for the development of the techniques of long-term storage of the strain studied.

Variant 3 of obtaining of dormant cells was relied on for the development of pseudomonad cultures on the surface of soil agar for 1 month with the subsequent cell transfer into the physiological saline. In this variant, we obtained coccoid forms, which differed morphologically from the vegetative cells and for which the CFU titer was 10^7 CFU/mL upon storage (1.5 months) in saline. It is important to point out that in variant 3 (on soil agar) dormant cells were formed in the cycles of culture development. These properties are necessary and sufficient to characterize the dormant cells of *P. fluorescens* 26K as specialized prokaryotic dormant forms [20].

Properties of the pseudomonad dormant cells. The properties of pseudomonad dormant forms were as follows: (a) long-term (three months) retention of the capacity for growth resumption; (b) increased resistance to heating (the variant with introduction of C_{12}-AHB) (Fig. 1) or drying (the variant of development on soil agar); (c) the respiratory activity not revealed experimentally (data not shown); and (d) the absence of signs of division and morphological differences from the vegetative cells (Fig. 2).

One of the indicators of diversity of the 26K dormant cells obtained in different variants was their different resistance to heating at 50, 55, and 60°C for 5 min. The thermal resistance of the dormant forms was determined as a decrease in the number of viable cells after heating relative to the control (the CFU number before heating) and was compared to vegetative cells. These modes of heat treatment caused all vegetative cells to die completely (Fig. 1, line *1*). The number of CFU in suspensions of the dormant cells of one storage term (two months) formed by addition of C_{12}-AHB (7×10^{-4} M) decreased by 1.5 orders of magnitude after heating at 50°C and up to undetectable values after heating at 55°C (Fig. 1, line *2*). An insignificant part of the population (0.001%) of the pseudomonad dormant cells formed at increased C_{12}-AHB concentration (1×10^{-3} M) was resistant to heating at 55°C, but not at 60°C (Fig. 1, line *3*). It was also noted that the pseudomonad cells formed when they developed on the soil agar surface (variant 3) were resistant to drying, which was determined by plating the aliquots of washings off the surface of dried out agar (1.5 months) and before drying out of the agar (0.5 months). However, they were not resistant to heating (the CFU number decreased to zero values).

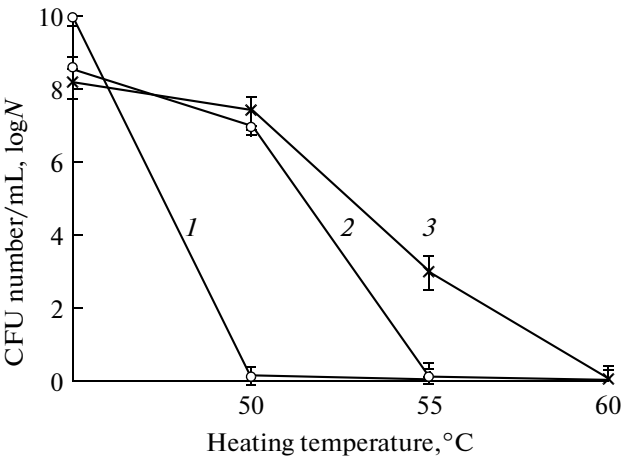

Fig. 1. Thermal resistance (heating at 50–60°C, 5 min) of (*1*) vegetative cells of the strain *P. fluorescens* 26K (LB broth, 24 h) and (*2, 3*) CLC (2 months of incubation) obtained upon introduction of C_{12}-AHB at a concentration of 77×10^{-4} M (*2*) and 1×10^{-3} M (*3*).

The electron microscopy examinations of *P. fluorescens* 26K dormant cells formed in the variants 2 and 3 (three months of incubation) revealed the specific features in their ultrastructure that allow these cells to be attributed to cyst-like dormant forms (Figs. 2, 3): (1) thickened cell envelopes; (2) altered cytoplasmic structure; and (3) condensed DNA in the nucleoid. At the same time, the intraspecies and intrapopulation diversity in the ultrastructural organization of pseudomonad cystlike dormant cells (CLC) was revealed.

In variant 2 suspensions (introduction of C_{12}-AHB, 7×10^{-4} M) were present CLC with (1) denser cell envelope; (2) increased periplasmic space; (3) lumpy cytoplasmic texture; and (4) condensed chromatin with especially compact structure sites (Figs. 2a–2c). Cell-like structures 0.2–0.3 μm in size encased in remnant envelopes of autolysed cells appeared to be absolutely unusual. They were characterized by (1) the presence of concentric thickened membrane layers with high electron density and (2) fine-grained cytoplasmic texture with low electron density and detectable DNA strands (Figs. 2d–2f).

Three types of cells were revealed in variant 3 CLC suspensions (incubation for 1.5 months in the saline of

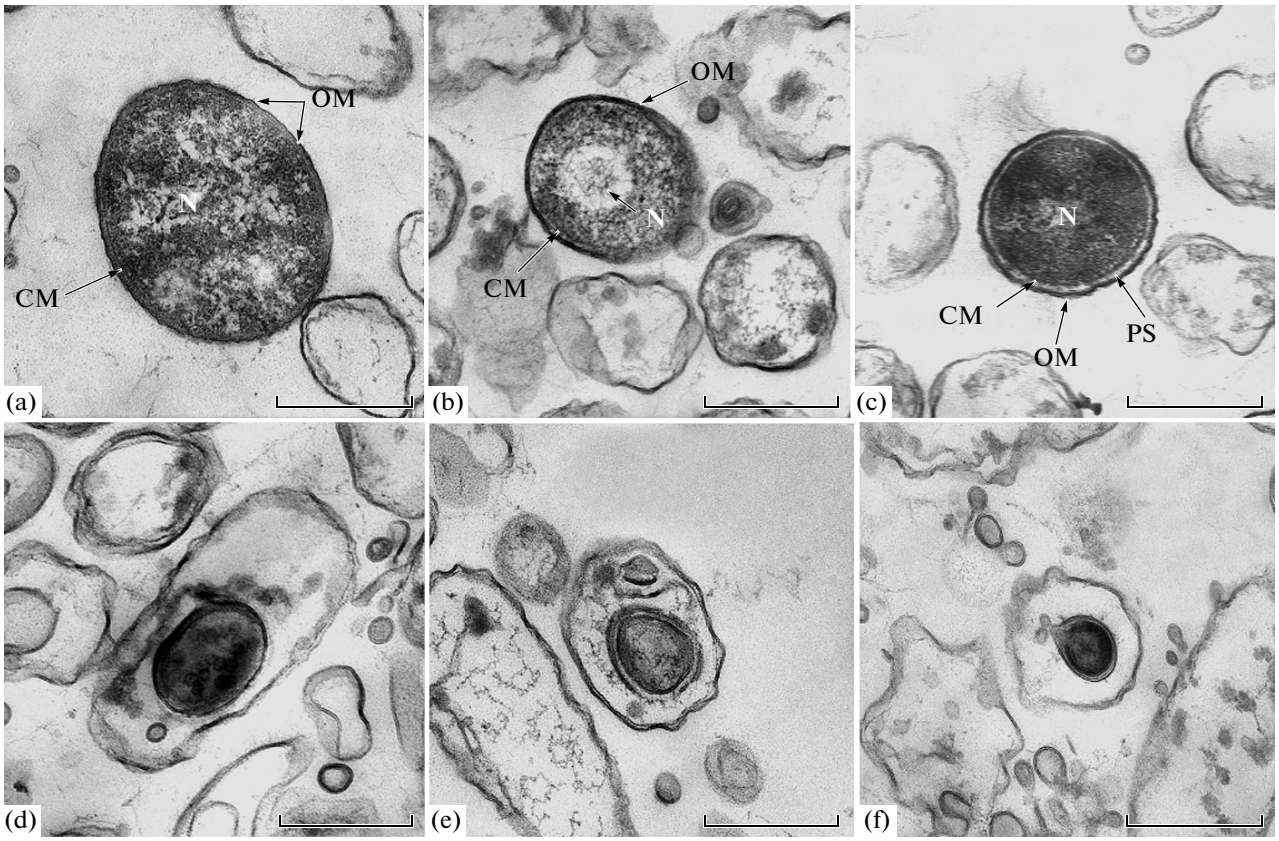

Fig. 2. Ultrastructural organization of the *P. fluorescens* 26K CLC obtained upon introduction into the stationary phase culture of C_{12}-AHB at a concentration of 7×10^{-4} M (a–f). Designations: OM, outer membrane; CM, cytoplasmic membrane; PS, periplasmic space; N, nucleoid. Scale bar is 0.3 μm.

cells washed from soil agar) (Fig. 3). The first morphological type was rod-shaped cells (0.7–0.8 × 0.4–0.5 μm) characterized by (1) thickened cell envelopes, especially at the cell poles; (2) detectable external loose electron-transparent capsular layer; (3) high electron density and homogeneous fine-grained texture of the cytoplasm; and (4) pronounced nucleoid condensation (Fig. 3a). The second CLC type was represented by rounded forms of submicrometer sizes (0.4–0.5 μm), which were characterized by (1) increased periplasmic space; (2) fine-granular cytoplasmic texture; and (3) well-defined compactness of the nucleoid (Fig. 3b). The third CLC type also revealed in the suspensions of this variant was characterized by (1) ultrasmall size (0.2–0.3 μm) and (2) the presence of thickened cell envelopes of uniform electron density and with the absence of lamellar structure (Figs. 3c, 3d).

When the CLC of *P. fluorescens* 26K were plated on solid media, phenotypic variability of the developing microbial population was observed, which was well diagnosed by morphological characteristics of grown colonies. For example, when the dormant cell cultures from the dominant variant (S-type) were obtained by addition of C_{12}-AHB (2.5×10^{-4} M and 7×10^{-4} M), emergence of other colony morphology variants was revealed. They were represented by (1) the M-type forming colonies with a mucous surface and a convex center; (2) the Sm-type with small colonies (d = 1–1.5 mm); and (3) the Tr-type forming semitransparent colonies with irregular edges. The rate of occurrence of the variants differing from the dominant type increased on prolonged storage of cultures (three months) (Fig. 4). The index of phenotypic variability also depended on the quality of plated CLC. Thus, heating of CLC (at 50 or 55°C, 5 min) before inoculation resulted in practically complete substitution of the M-type for the dominant S-type. Predominance of the M-type (88%) was also noted in the populations grown on LB agar with the germination of the CLC (1.5 months) obtained by cell washout from soil agar into saline.

P. fluorescens 26K variants differed in the stability of their colony morphology after sequential transfers onto solid media (LB agar). The least stable was the Tr-type variant completely reverting to the dominant S-type in the first transfer; among the variants of the Sm- and M-types, reversion to the dominant phenotype was observed in the second or third transfer (data not shown).

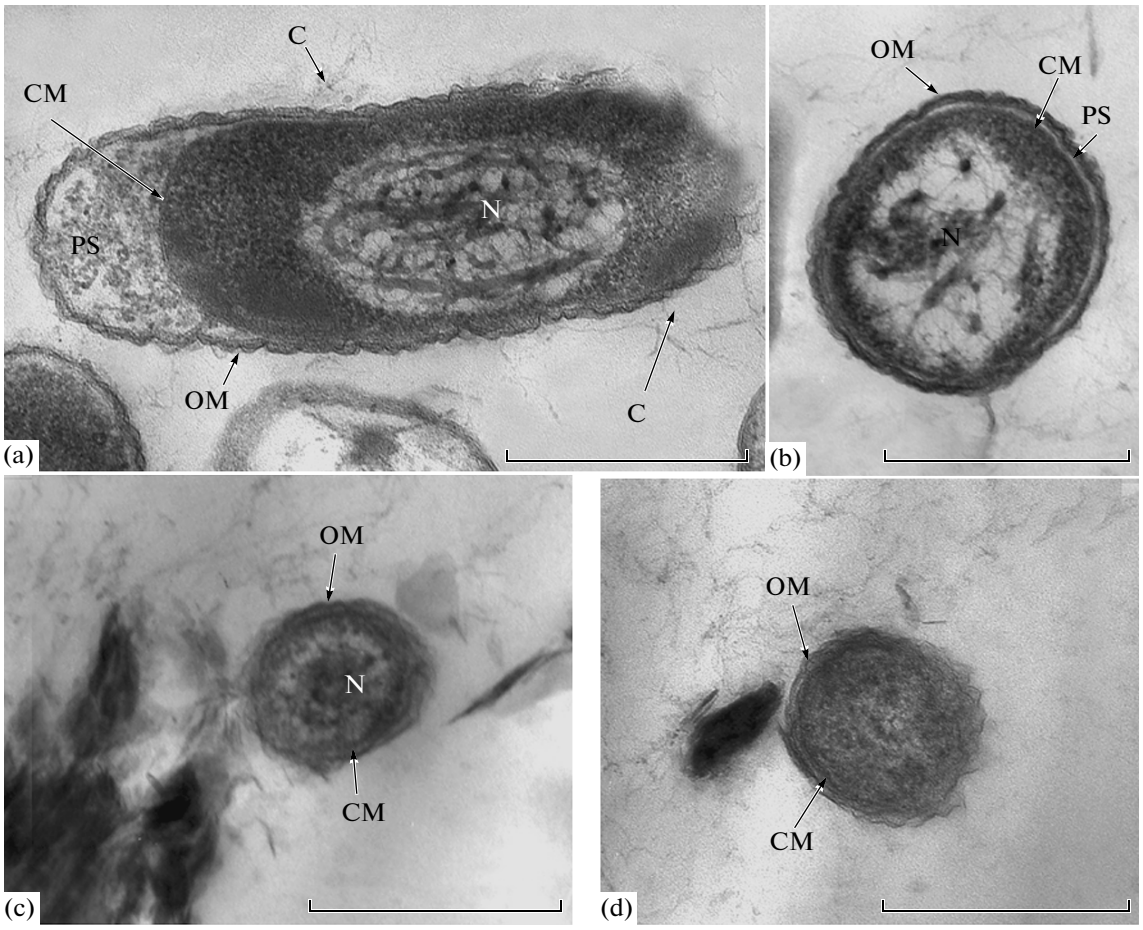

Fig. 3. Ultrastructural organization of the *P. fluorescens* 26K CLC of different morphotypes obtained when the bacterium developed on soil agar. Designations: OM, outer membrane; CM, cytoplasmic membrane; PS, periplasmic space; N, nucleoid; C, capsule. The bar scale is 0.3 mm.

Thus, the dormant state of *P. fluorescens* 26K cells (in the form of CLC) is associated with an increase in intrapopulation variability, which is considered to be one of the mechanisms of their adaptation to the changing environmental conditions.

The pseudomonad strain studied was resistant to antimicrobial agents. For example, when kanamycin at a concentration of 10 μg/mL was introduced into *P. fluorescens* 26K culture at the active growth phase (in LB broth), the CFU titer did not change (plating on LB agar and LB agar with kanamycin, 10 μg/mL), remaining at $2–3 \times 10^9$ CFU/mL. No qualitative and quantitative changes were revealed in the spectrum of the colony morphology variants by platings on the antibiotic-containing selective medium: the M-phenotype predominated (85–95%) on LB agar with and without kanamycin (10 μg/mL). *P. fluorescens* 26K cells were also resistant to other antibiotics: a significant growth-inhibiting effect of kanamycin was observed only at a concentration of 200 μg/mL; that of tetracycline, at 100 μg/mL.

In view of increasing phenotypic variability in populations grown from dormant forms of the strain studied, it was of special interest to study the adaptive possibilities of the cultures developing upon inoculation with vegetative cells or dormant forms in relation to xenobiotics.

Growth of *P. fluorescens* 26K on aromatic substrates. The capacity of *P. fluorescens* 26K for adaptation to aromatic substrates was determined as growth of the bacteria after nine transfers for the cells developed from the germinated dormant forms and for ten transfers for the vegetative cells of the initial culture. The compounds, which are normally poorly degraded by microorganisms either due to their toxicity (e.g., 2-CP, trichlorophenols, PCP) or, not being toxic, require a special set of enzymes for utilization (e.g., *p*-toluate, 2,6-DCB) were used [21–24]. In our experiments, the growth of pseudomonads on agar medium was observed on all the substrates, irrespective of whether the inoculum used was obtained by sequential transfers of cells developing from dormant forms (called "inoculum from dormant forms," or

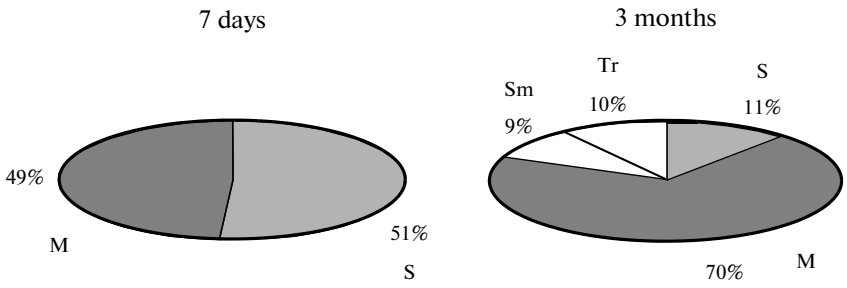

Fig. 4. The phenotype variability index of the populations grown upon germination of anabiotic dormant cells (C_{12}-AHB, 2.5×10^{-4} M) stored for 7 days and 3 months.

"IDF") or from vegetative cells of the initial culture (hereinafter IVF) (table). In the variants of inoculation with cultures after dormancy, surface growth was better only on 8 of the 24 substrates used.

Pseudomonads did not grow in liquid media with more toxic di- and polychlorophenols (as the sole growth substrates) in both variants (with the use of IDF and IVF). Spectrophotometric measurements did not show any loss of the introduced toxicants (data not shown). In this respect, the strain *P. fluorescens* 26K differed from the strain *R. opacus* 1cp, whose cells after the state of dormancy appeared to be capable of utilizing all the chlorophenols tested as growth substrates, although to a different degree [8].

The substrates which degradation is usually not difficult for active degrader strains, such as benzoate, gentisate, and PHB, supported the growth of pseudomonads in both IDF and IVF variants equally well, although they required inductions of the relevant enzymes for complete utilization. No significant lag phase was observed when PHB or gentisate were used as growth substrates (Figs. 5a, 5b). The growth characteristics of the cultures of both inoculation types differed insignificantly when they grew on PHB: for the IDF variant, the specific growth rate (μ) was 0.0092 h^{-1}, and the duplication time (t_d) was 75 h; for IVF, $\mu = 0.0089$ h^{-1} and $t_d = 78$ h. On gentisate, the values of these parameters were the same for both variants: $\mu = 0.0198$ h^{-1}; $t_d = 35$ h. The highest biomass increment was observed when the cultures grew on gentisate.

Comparative study of the growth of the cultures developing from IDF and IVF on benzoate and 3-CB showed that in both cases an insignificant lag phase (up to 10 h) was observed. The parameters of growth of *P. fluorescens* 26K cultures on benzoate were $\mu = 0.03$ h^{-1} and $t_d = 23$ h for both inoculation types. When the cultures of *P. fluorescens* 26K grew on 3-CB, the growth characteristic were the same for the IDF and IVF variants: $\mu = 0.018$ h^{-1}; $t_d = 39$ h. The results obtained were expectable, since it is known that the introduction of an additional chlorine atom into the substrate molecule significantly increases its resistance to a microbial attack, and the degradation of 3-CB requires induction of the enzymes which differ in substrate specificity from the enzymes involved in the degradation of the nonchlorinated substrate [17].

In the cell-free extracts of the pseudomonads grown from IDF and IVF and developing on benzoate and 3-CB, the activities of the key enzymes of degradation of these compounds were determined. The activity of catechol 1,2-dioxygenase (PCat 1,2-DO) in the benzoate-grown cultures of both inoculation types was practically the same: 0.0331 (IDF variant) and 0.0236 U/mg protein (IVF variant), respectively. The activity of Cat 1,2-DO with 4-chlorocatechol (4CPC) did not exceed 7%; it was even lower with other chlorinated substrates, which is in agreement with the expected Cat 1,2-DO activity of the ordinary *ortho*-pathway, which is characterized by narrow substrate specificity in most of the bacteria described and is not capable of oxidizing of chlorinated substrates [17]. Of the greatest interest is the discovery of enzymatic activity in relation to 5-chloromuconolactone (5CML) in both extracts. It is normally detected only as a function of chloromuconate cycloisomerase (CMCI), which catalyzes the formation of *trans*-dienelactone from 5CML in gram-negative bacteria [25]. In *R. opacus* 1cp, the formation of *cis*-dienelactone from 5CML occurred under the action of a special enzyme, chloromuconolactone dehalogenase (CMLD) [26]. However, it is known that both types of enzymes, CMCI of gram-negative bacteria and CMLD of the strain 1cp, are only induced when the bacteria grow on chlorinated substrates. Muconatecycloisomerases (MCI) of the ordinary *ortho*-pathway induced when grown on benzoate are not active with 5CML. Thus, the presence of CMCI in benzoate-grown *P. fluorescens* cells requires further investigation.

When strain 26K cultures grew on 3-CB, the activities of all the enzymes of the modified *ortho*-pathway—chlorocatechol 1,2-dioxygenase (CCat 1,2-DO), CMCI, and dienelactone hydrolase (DLH)—were revealed in the cell-free extracts of both inoculation variants. The activity of CCat 1,2-DO with 4-CCat was about 30% of the level of activity with Cat; the activity in relation to methylcatechols was also low, up to 24%. The DLH activity was also higher

with 2-chlorodienelactone than with the unsubstituted substrate (124 and 100%, respectively).

In the cell-free extracts of the cells grown on 3-CB and the cells grown on benzoate, an increase in the optical density at 280 nm was observed when 5CML was used as a substrate. This enzymatic reaction may be catalyzed by CMCI, an enzyme characteristic of the pathway of utilization of chlorinated substrates and converting 2-chloromuconate to one of the dienelactone isomers. However, the activity with 5CML was observed in the fraction which did not bind to the carrier Q-Sepharose when the cell-free extract was applied on the column, while CMCI is eluted from the carrier of this type significantly later. The patterns of CMLD induction are also revealed in the cells of the cultures of both inoculation types and do not indicate the presence of any differences in the set of enzymes or the enzymatic activity between the cultures inoculated with the vegetative cells and the cultures after the state of dormancy.

Efficient degradation of *p*-toluate may be carried out by the bacteria having the enzyme sets differing from those involved in the degradation of both unsubstituted and chlorinated substrates [21, 22]. At present, only several bacterial strains are known, which are able to completely utilize this compound due to an additional enzyme, methylmuconolactone methylisomerase, catalyzing the transfer of a methyl group in the aromatic group. Experiments showed that the strain *P. fluorescens* 26K grew in liquid medium on *p*-toluate (Fig. 5c) after a significant lag period (6 days). After that, an insignificant increase in optic density, diauxy, and a secondary OD increase were observed in both inoculation variants. The culture growth parameters differed somewhat: the specific growth rate was insignificantly higher and the duplication time was shorter in the IDF variant ($\mu = 0.00484\ h^{-1}$; $t_d = 143$ h) than in the culture inoculated with the vegetative cells ($\mu = 0.00391\ h^{-1}$; $t_d = 177$ h).

The strain *P. fluorescens* 26K also grew better on the medium with 2,6-DCB in the presence of succinate (Fig. 5d) in the IDF variant ($\mu = 0.0034\ h^{-1}$; $t_d = 204$ h) than in the IVF variant ($\mu = 0.0022\ h^{-1}$; $t_d = 315$ h). Diauxy was noted in this variant, similar to growth on *p*-toluate. The character of dynamics of the development of the culture on toluate and 2,6-DCB allows a suggestion that growth arrest before its resumption (diauxy) is linked to the replacement of the phase variant, which more actively cleaves the substrate in the cultures inoculated with cells after dormancy with a less active variant, which, however, is possibly more resistant to the presence of toxicants. Although on the whole, the growth parameters were very low in the cultures of both inoculation types, it should be kept in mind that both *p*-toluate and 2,6-DCB are difficultly degradable substrates. That is why even insignificant differences in the growth characteristics in the intrapopulation variants may be advantageous for cell survival under unfavorable conditions.

Assessment of culture growth on agarized medium according to a three-point scale

Growth substrate	Growth (score) after inoculation with suspensions of cells grown after the transfers of cultures	
	DF (CLC)	Vegetative cells
2-CP	2	1
2,3-DCP	2	1
2,4-DCP	1	1
2,5-DCP	1	1
2,6-DCP	1	1
3,4-DCP	1	1
2,3,4-TCP	1	1
2,4,5-TCP	1	1
2,4,6-TCP	1	2
PCP	2	2
2-CB	2	1
3-CB	1	1
4-CB	2	1
2,4-DCB	2	2
2,5-DCB	3	2
2,6-DCB	3	2
3,5-DCB	2	2
PHB	3	3
Gentisate	3	3
Benzoate	3	3
Salicylate	2	2
5-Chlorosalicylate	2	1
p-Toluate	2	1
Phenol	1	1

Thus, our experiments showed that the xenobiotic-degrading strain *P. fluorescens* 26K forms dormant cells, which retain long-term viability, possess increased resistance to unfavorable effects, and are characterized by specific ultrastructural organization. The use of *P. fluorescens* 26K inoculum obtained after the transfer of the germinated dormant forms did not exert a strong influence on the improvement in the degrading activity of the developing cultures in relation to a broad range of xenobiotics. It can not be excluded that this is primarily associated with the instability of the minor intrapopulation variants, which quickly revert to the dominant phenotype under the laboratory experimental conditions. It should be noted that the level of extracellular autoregulatory factors controlling phase transfers seems to be insufficient for the stable maintenance of a certain phenotype

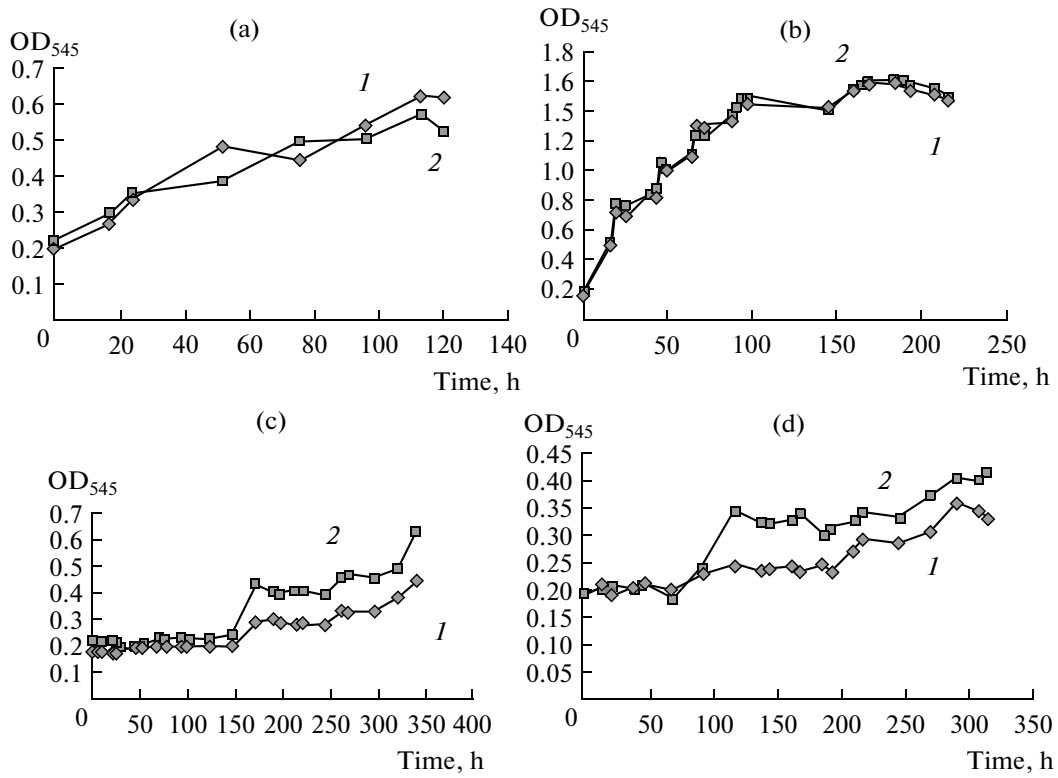

Fig. 5. Growth curves of *P. fluorescens* 26K cultures grown upon inoculation with vegetative cells (*1*) and dormant forms (*2*): growth in medium with PHB (a); gentisate (b); *p*-toluate (c); and 2,6-DCB (d).

(supposedly, with an increased biodegrading activity) in the test cultures with low initial cell density. At the same time, in certain loci in the soils where bacteria develop in the form of microcolonies or biofilms, the level of these autoregulators may be substantially higher than in the laboratory cultures. Therefore, the development of the conditions, which are more adequate to natural ones, appears to be expedient for the search for the variants with improved adaptability and the capacity for the utilization of xenobiotics.

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