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EXPERIMENTAL ARTICLES

Structural and Physiological Diversity among Cystlike Resting Cells of Bacteria of the Genus *Pseudomonas*

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Abstract—Cystlike resting cells (CRC) of non-spore-forming gram-negative bacteria of the genus *Pseudomonas, P. aurantiaca* and *P. fluorescens,* were obtained and characterized for the first time; their physiological and morphological diversity was demonstrated. The following properties were common for all the revealed types of CRC as dormant forms: (1) long-term (up to 6 months or longer) maintenance of viability in the absence of culture growth and cell respiration; (2) absence of an experimentally detectable level of metabolism; (3) higher resistance to damage and autolysis under the action of provoking factors than in metabolically active vegetative cells; and (4) specific features of ultrastructural organization absent in vegetative cells: thickened and lamellar envelopes, clumpy structure of the cytoplasm, and condensed DNA in nucleoid. The differences in various types of CRC concern the thickness and lamellar structure of cell envelopes, as well as the presence and thickness of the capsular layer. In particular, forms ultrastructurally similar to typical bacterial cysts were revealed in pseudomonad populations growing on soil agar. Physiological diversity was revealed in different levels of viability preservation and thermal resistance in various types of CRC and depended on the conditions of their formation. The optimal conditions and procedures for obtaining *P. aurantiaca* and *P. fluorescens* CRC that retain the ability to form colonies on standard nutrient media are as follows: (1) a twofold decrease of nitrogen content in the growth medium; (2) an increased level of anabiosis autoinducer (C_{12}-AHB, 10^{-4} M) in stationary cultures; (3) transfer of the cells from stationary cultures to a starvation medium with silica; (4) cultivation in soil extract; and (5) development of cultures on soil agar. The CRC from the cultures grown in soil extract or starvation medium with silica proved to be resistant to heat treatment (60°C, 5 min). In the CRC formed in nitrogen-limited media, the degree of heat resistance increased at longer incubation (1.5 to 6 months). CRCs on soil agar surface were resistant to desiccation. The ultrastructure of the morphologically varied types of *P. aurantiaca* CRC formed under simulated natural conditions is described for the first time. The data on the intraspecies diversity of pseudomonad dormant forms contribute to the concept of plasticity of the life style and adaptive reactions that ensure survival of these bacteria in unfavorable environmental conditions.

Key words: dormancy, cystlike cells, anabiotic cells, stress resistance, polymorphism of dormant forms, anabiosis autoinducers, alkyl hydroxybenzenes.

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The genus *Pseudomonas* includes the species of non-spore-forming gram-negative saprotrophic, pathogenic, and conditionally pathogenic bacteria widespread in soils, silts, and aquatic habitats [1, 2]. The members of this genus possess extensive metabolic potential and play an important role in the biospheric processes. However, the ways and forms of long-term survival of these bacteria in conditions unfavorable for growth and proliferation as well as preservation of viability are poorly known. Like other non-spore-forming bacteria, pseudomonads have no specialized dormant forms that appear as a result of cell differentiation at the final stages of the ontogenetic development cycle of microbial cultures [1, 2]. Transition into a non-culturable state is believed to be one of their strategies for survival in conditions unfavorable for growth (starvation) [3–5]. However, the capacity of pseudomonads for long-term survival in soils and laboratory microcosms with subsequent reproduction without any special procedures of revival (resuscitation) suggests the existence of other mechanisms of dormancy. Heterogeneity of the dormant pseudomonad populations (coexistence of culturable, viable non-culturable, and dormant inactive cells in different ratios) was shown in the study of persistence of introducers, including marker strains *P. fluorescens* CHA0 in soil [5] and *P. fluorescens* DR-54 BN14 in barley rhizosphere [6].

Long-term survival of pseudomonades under natural conditions is probably ensured mainly by transition of cells to a resting anabiotic state. For example, dried samples of soil enriched by a *P. fluorescens* culture

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were shown to contain cystlike cells of these bacteria, probably possessing the properties of dormancy [7]. Afterwards, Zechman and Casida, on the basis of soil sample analysis, inferred that pseudomonads form stable, long-preserved resting cystlike cells in soil [8]. Direct evidence of the ability of pseudomonads to form resting cells intended for long-term survival and maintenance of a species in their development cycle was obtained in later studies. They showed that formation of cystlike cells (CRC) of *P. carboxydoflava* is induced by an increased concentration of extracellular autoregulatory factors d_1 (autoinducers of anabiosis) and their analogues [9, 10], derivatives of alkyl hydroxybenzenes (AHB) [11]. However, the titer of formed CRC was low. Evidently, special conditions are required for CRC to be formed by pseudomonads in the cycle of their culture developmen.

The goal of this work was to select optimal conditions for formation of specialized resting cells by pseudomonads and to characterize the structural and functional diversity of CRC, their depth of dormancy, ability to reverse to growth, and resistance to stress factors.

MATERIALS AND METHODS

Objects of research were collection strains of two bacterial species of the genus *Pseudomonas: P. aurantiaca* VKM B-1558 and *P. fluorescens* NCIMB 9046.

Media and cultivation conditions. The cultures of strains B-1558 and NCIMB 9046 were grown in 50% Luria–Bertani (LB) broth, M9 medium with glucose (2 g/l), and a deficient synthetic medium containing the following (g/l): glucose, 2; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.2–0.5; $K_2HPO_4 \cdot 3H_2O$, 1; $CaCl_2$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; yeast extract (Difco), 0.05; trace elements (mg/l): $FeSO_4 \cdot 7H_2O$, 20; $MnCl_2 \cdot 4H_2O$, 20; $ZnSO_4$, 0.4; $B(OH)_3$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.5; and $Na_2MoO_4 \cdot 2H_2O$, 0.2; pH after sterilization, 7.25. The bacteria were cultivated at 28°C in 250-ml flasks (50 ml of the medium) or in 20-ml test tubes with 2 ml of the medium in a shaker at 140–160 rpm. Stationary phase cultures grown in nutrient broth were used as inoculum. The inoculum was introduced in amounts that yielded the initial optical density 0.2 (λ = 540 nm, l = 10 mm).

Cystlike resting cells (CRC) of pseudomonads were obtained in aging cultures grown in nitrogen-deficient media (two to five times less than in the M9 medium with 1 g of $(NH_4)_2SO_4$/l) and incubated for a long time (up to six months) at room temperature. Anabiotic cells were obtained by supplementing the stationary cultures (48 h of growth) with the chemical analogue of microbial anabiosis autoinducers, C_{12}-AHB (M = 194), as ethanol solutions to the final concentrations of 5×10^{-5}, 1×10^{-4}, 2.5×10^{-4}, and 5×10^{-4} M; the final ethanol concentration did not exceed 0.5% (vol/vol). Other variants of obtaining CRC included incubation of the cultures for 3 months on soil agar, in aqueous extract from soils, or in 0.09% solution of sodium silicate $Na_2SiO_3 \cdot 9H_2O$ (pH 7.4). Soil agar and water soil extract were prepared using air-dried meadow soil taken from the floodplain of the Oka River (Pushchino, Moscow oblast), which was ground in a mortar, sieved, and suspended in $K_2HPO_4 \cdot 3H_2O$ solution (0.5 g/l, pH 7.2) at 1 : 2.5 (wt/vol). Agar (1.7%) was added to one part of soil suspension, while the other part was filtered to remove suspended particles and to obtain the aqueous extract. Soil agar and extract were autoclaved twice at 1 atm (30 min). The pseudomonad culture was grown in the medium with twofold nitrogen limitation to the early stationary growth phase and introduced into test tubes with soil extract or sodium silicate solution in a ratio of 1 : 10 (vol/vol) and to the surface of soil agar (0.1 ml) and incubated for one to three months at room temperature.

Microbiological methods. Cell viability was determined by counting the colony-forming units (CFU); cell suspensions in appropriate tenfold dilutions were plated on nutrient agar and incubated at 28°C for 3 days. In some experiments, 0.15 ml of sodium pyruvate solution was applied to the surface of a dish with the medium (to the final concentration 0.1% of the medium volume) as recommended [12]. At a low or undetectable CFU titer in long-stored cultures, cell viability was estimated using the most probable number method (MPN). Heat resistance was determined by counting the cells that remained viable after heating of cell suspensions (0.7 ml) in an ultrathermostat at 50–70°C for 5 min. Respiration activity was detected in a LP7E polarograph (Czech Republic) in a 1-ml oxygen cell.

Microscopic examinations were carried out using a Reichert microscope (Zatopan, Austria) equipped with a phase-contrast device. Vegetative and resting pseudomonad cells were stained with a two-component dye, Live/Dead BacLight kit (Molecular Probes Inc.; www.probes.com), according to the manufacturer's instructions. Stained preparations were examined in an epifluorescence microscope (Axioplan, Carl Zeiss, Germany) with a 100×1.3 objective. For cell counting, at least 20 fields of vision were examined. Electron microscopic studies were carried out in accordance with the methods described previously [13].

Repeatability of measurements in the experiments was threefold with three independent series of experiments. The presented results show the averaged values. The data were statistically analyzed by the Student's test, with the criterion of probability $P < 0.05$ taken as sufficient.

RESULTS

Conditions for Formation of Pseudomonad Cyst-like Resting Cells

It was shown that rich nutrient medium (50% nutrient broth or complete M9 medium) used for cultivation of pseudomonades did not promote formation of cyst-

like cells: in the course of long-term incubation and aging of the cultures, the number of viable cells (CFU/ml) in the cultures of strains B-1558 and NCIMB 9046 decreased monotonously to single CFU/ml by month 2.5 (Fig. 1a). At the same time, the titer of viable resting cells of pseudomonads was reliably high in other cultivation conditions that we revealed (variants 1–5).

Variant 1. In this variant, CRC were obtained using a nutrient medium modification reproducing the frequent ecological situation of nitrogen deficiency and providing for a C > N unbalance with nitrogen source limitation, which had been shown previously [14] to intensify biosynthesis of factor d_1 (AHB), an autoinducer of anabiosis. In this variant of medium modification, the efficiency of CRC formation and the ability of those resting to reverse to growth depended on the degree of N limitation. Thus, after three weeks, 40–50% of the post-stationary cells of strains B-1558 and NCIMB 9046 grown in the medium with twofold reduced nitrogen content were shown to develop the refractivity of cells (Figs. 1c, 1e) not found in stationary cultures (Fig. 1b). The number of cells retaining the colony-forming ability decreased within one order of magnitude in a month and stabilized at a level of 7×10^6–3×10^7 CFU/ml (7–20% of the initial level) at further incubation for up to 6 months (Fig. 1a). Weakly refractory cells of pseudomonads were formed in the medium with fivefold decreased N content; however, there was an abrupt decrease in CFU number by four orders of magnitude (to 3×10^4 CFU/ml) after a month of incubation and to single CFU after two months (Fig. 1a). The data from Figure 1a show that for strains B-1558 and NCIMB 9046 the medium with a twofold reduced nitrogen content is optimal for the formation of CRC retaining their growth potential for a long time.

Variant 2. Another method of obtaining anabiotic pseudomonad cells simulated the situation with soil drying and, as a consequence, an increase of the concentration of autoregulators by adding C_{12}-AHB (the chemical analogue of microbial anabiosis autoinducers) in the concentrations of 5×10^{-5} to 5×10^{-4} M to the cultures of early stationary phase grown in complete M9 medium. In 15 min after the addition of C_{12}-AHB in concentrations of 1×10^{-4}–2.5×10^{-4} M or 5×10^{-5} M–2.5×10^{-4} M to the cultures of strains B-1558 and NCIMB 9046, respectively, refractivity developed in 40–85% of the cells depending on the AHB concentration. In 1.3 month, the number of CFU in experimental cultures was higher than in the control variants of incubation in M9 medium (Figs. 2a, 2b, curves *1*). For obtaining refractory cells retaining the highest viability (38–45% of the initial CFU titer before the beginning of storage), it was optimal to introduce C_{12}-AHB in the concentrations 10^{-4} M for strain B-1558 (Fig. 2a, curve *2*) and $(1.0–2.5) \times 10^{-4}$ M for strain NCIMB 9046 (Fig. 2b, curves *2* and *3*). The colony-forming ability of anabiotic cells decreased in the dynamics of incubation of B-1558 cultures in the medium with an increased

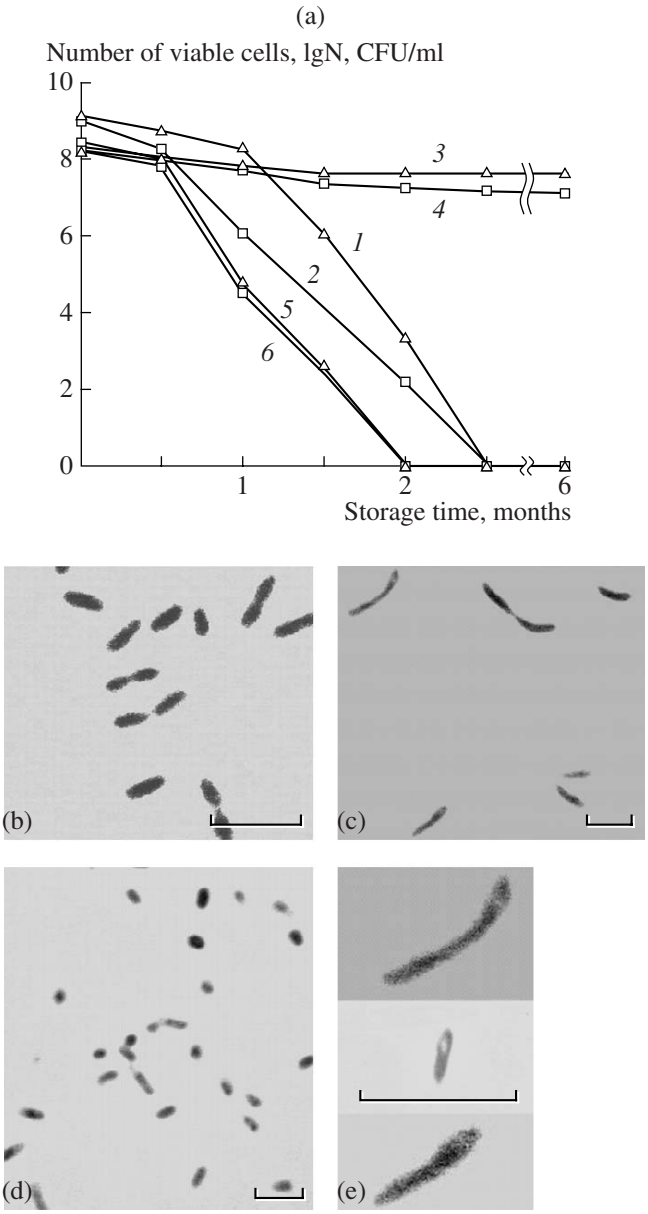

Fig. 1. a, The number of colony-forming cells (CFU/ml) in long-incubated cultures of *P. aurantiaca* B-1558 *(1, 3, 5)* and *P. fluorescens* NCIMB 9046 *(2, 4, 6)* grown in 50% nutrient broth *(1, 2),* the medium with twofold nitrogen limitation *(3, 4),* and the medium with fivefold nitrogen limitation *(5, 6).* Characteristic value of deviations from the mean CFU value (taking into account presentation of the data in logarithmic scale) is indicated by the line at the side. Vegetative (b) and resting (c–e) cells of *P. aurantiaca* B-1558 in phase-contrast microscope. Variants of obtaining the dormant forms: (c, e) in cultures grown in the medium with twofold nitrogen limitation; (d) incubation on soil agar. Scale bar is 2 μm.

C_{12}-AHB concentration of 2.5×10^{-4} M (Fig. 2a, curve *3*). The increase of C_{12}-AHB content to 5×10^{-4} M caused the formation of cells that showed no colony-forming ability when plated on standard nutrient media (Fig. 2a, curve *4*) but maintained external morphologi-

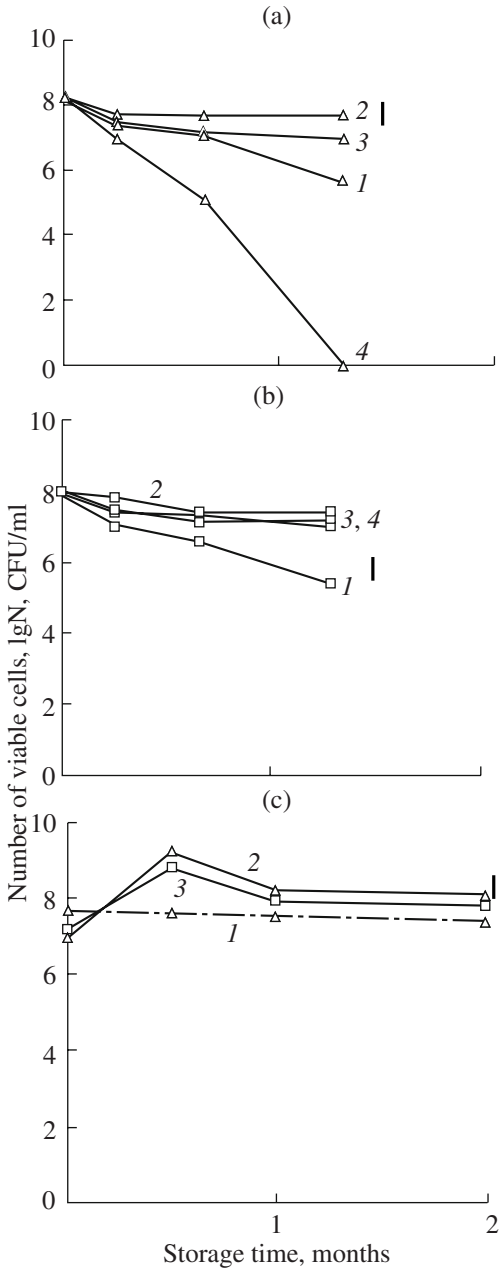

Fig. 2. The number of viable cells (CFU/ml): (a), *P. aurantiaca* B-1558 obtained at introduction of C_{12}-AHB in the following concentrations: *(2)* 1×10^{-4} M; (*3*) 2.5×10^{-4} M; (*4*) 5×10^{-4} M; (b) *P. fluorescens* NCIMB 9046 at introduction of C_{12}-AHB: (*2*) 5×10^{-5} M; and (*3*) 1×10^{-4} M; (*4*) 2.5×10^{-4} M. In the control variants (*1* on graphs a, b) C_{12}-AHB was not introduced; (c) in suspensions of *P. aurantiaca* B-1558 (*1, 2*) and *P. fluorescens* NCIMB 9046 (*3*) in (*1*) 0.09% silica solution or (*2, 3*) soil extract. Characteristic value of deviations from the mean CFU value is indicated by the line at the side.

cal integrity. Thus, the formation of viable anabiotic cells by both pseudomonad strains occurred only in a narrow range of concentrations of introduced C_{12}-AHB.

Other methods of obtaining resting forms in laboratory conditions also simulated ecological situations.

Variant 3 was based on the transfer of cells from pseudomonad stationary cultures grown in the medium with twofold N limitation into 0.09% $Na_2SiO_3 \cdot 9H_2O$ solution with subsequent incubation at room temperature for two months. Suspensions of B-1558 cells incubated under these conditions had a high titer of viable cells, from 35–47% of the initial number (Fig. 2c, curve *1*); the cells were coccoid in shape. The same data were obtained for strain NCIMB 9046.

In **variant 4,** the cells from similarly grown stationary cultures of strains B-1558 and NCIMB 9046 were inoculated into soil extract. After incubation for 14 days, there was a definite increase of CFU number (Fig. 2c, curves *2* and *3*), probably associated with cell proliferation due to utilization of the available amount of soil substances as a substrate. Further incubation of the pseudomonad suspensions resulted in a decrease (by the end of the first month) and subsequent stabilization of the number of viable cells at a level of 10^8 CFU/ml.

Finally, **variant 5,** providing for the development of B-1558 cultures on soil agar surface for 0.5–1 month, yielded morphologically different cells as coccoid forms, constituting 35–40% of the total cell number (Fig. 1d).

The Physiological Diversity of Pseudomonad CRC Depending on The Method of Their Production

The common properties of obtained CRC as dormant forms of the strains under study were as follows: (1) preservation of the ability for growth renewal for a long time (Figs. 1a, 2); (2) resistance to starvation and autolysis under provocative conditions (growth medium, temperature 20°C); (3) higher resistance to heating (Fig. 3); (4) respiration activity not revealed experimentally (polarographic measurements, data not shown); (5) morphological differences from the vegetative cells (Figs. 1b–1e, 4); and (6) formation in the cycle of culture development. These properties are necessary and sufficient for the characterization of pseudomonad CRC as specialized bacterial resting forms [13].

Physiological diversity of obtained CRC manifested itself in the differences in viability and heat resistance. The quickest stabilization (in 1 month) of CFU numbers obtained on standard nutrient media (35–45% of the CFU in the stationary phase) was observed for the CRC of both strains obtained in variant 2 (C_{12}-AHB, 10^{-4} M) and variant 3 ($Na_2SiO_3 \cdot 9H_2O$, 0.09%). The number of colony-forming CRC obtained in variant 1 (twofold nitrogen limitation) stabilized later, after 2–2.5 months of incubation, and remained at a high level of 7–20% (Fig. 1a). For 6-month suspensions of the same variant (strain B-1558), differential counting of "live" and "dead" cells was carried out (using the

Live/Dead test); the portion of colony-forming cells was found to be 21% of the total quantity of "live" cells with green fluorescence (Table).

Examination of the preparations of two-month cultures of strain B-1558 grown in the medium with fivefold nitrogen limitation showed greater heterogeneity of the cells. Besides the cells with brightly green and red fluorescence (i.e., live and dead), there were yellow-green, yellow-red, and yellow cells that seemed to possess an altered barrier function for penetration of the dye, propidium iodide. Although the number of live cells amounted to 10^8 cells/ml (Table), most of them lacked colony-forming ability (1–2 CFU/ml) on a standard medium or on the same medium with 0.1% sodium pyruvate. The MPN method showed a low titer of cells capable of growth renewal in a liquid medium (10^3 ml^{-1}), which was at least 5 orders of magnitude less than their total quantity and the quantity of "live" cells detected microscopically (Table).

Resistance to heating at 60°C for 5 min was another investigated aspect of the physiological diversity of strain B-1558 CRC. This treatment resulted in complete death of vegetative cells (Fig. 3a). The resistance of CRC to heating was determined as a decrease of the number of viable cells relative to the control. The CFU number in CRC suspensions incubated for the same period of time (1.5 months) in soil extract (variant 4) and in the starvation medium with $Na_2SiO_3 \cdot 9H_2O$ (variant 3) and obtained upon introduction of 1×10^{-4} M C_{12}-AHB (variant 2) decreased by four orders of magnitude (Fig. 3a). It was noted that plating the CRC suspensions of variant 3 resulted in CFU values higher by an order of magnitude when the solid medium was supplemented with sodium pyruvate; at the same time, the control variant (CRC without heating) showed no increase of CFU number due to pyruvate utilization (Fig. 3b).

CRC obtained in variant 1 (with twofold N limitation) and also stored for 1.5 months were less heat-resistant: the titer of their viability decreased by 7 orders of magnitude. Heat treatment at 50°C or 55°C for 5 min proved to be more sparing for them: the CFU number decreased by 1 and 2 orders of magnitude, respectively, compared to 3 and 5 orders of magnitude in vegetative cells (the graphs are not presented). However, at extension of incubation periods for the cultures of this variant to 6 months, CRC heat resistance significantly increased and CFU number after thermal treatment (60°C, 5 min) decreased by only 2 orders of magnitude (Fig. 3a).

It was also noted that CRC formed in variant 5 (development on soil agar surface) were resistant to desiccation and had a high colony-forming ability (60% of the initial one), which was determined by inoculation of aliquots of washouts from the surface of dry agar (3 months) and before drying (0.5 months). However, the degree of their heat resistance (60°C, 5 min) was not high (CFU number decreased by 6 and more orders of magnitude).

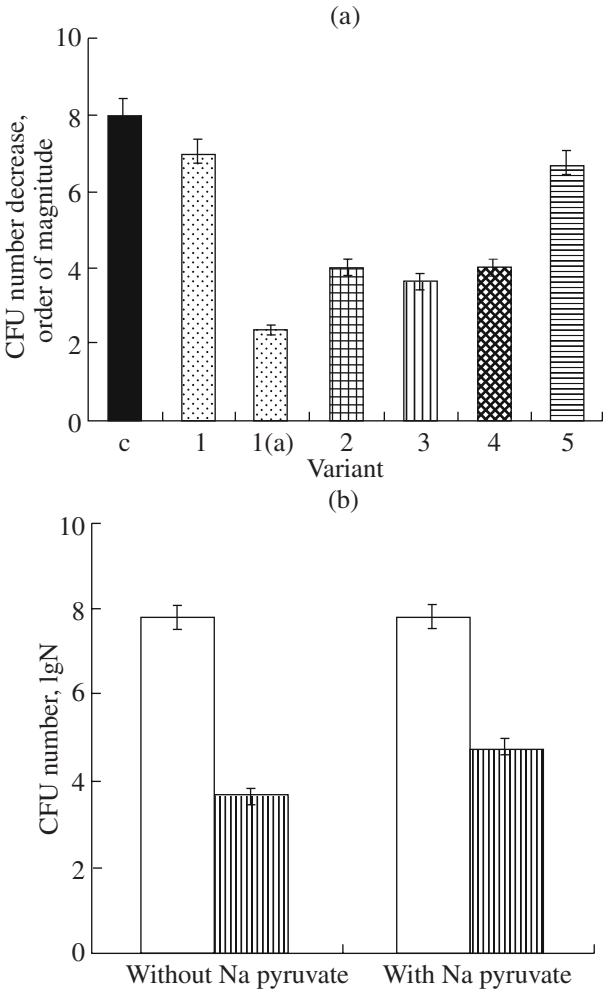

Fig. 3. a, Decrease of viability of vegetative cells and CRC of *P. aurantiaca* B-1558 after heat treatment at 60°C (5 min). Numerical designations (corresponding to the variants of CRC obtaining) are as follows: c, vegetative cells; 1, CRC (double N limitation, 1.5-month incubation); 1(a), CRC (double N limitation, 6-month incubation); 2, CRC with C_{12}-AHB (1×10^{-4} M); 3, CRC in Na_2SiO_3 solution; 4, CRC in soil extract; 5, CRC in wash-out from soil agar. b, Viability (CFU/ml) of CRC from variant 3 (in Na_2SiO_3 solution) before and after heating (60°C, 5 min) determined at inoculation on solid media with and without sodium pyruvate. Light columns, without heating; dark columns, after heating.

Morphological Diversity of the CRC of P. aurantiaca B-1558

Comparative electron microscopic analysis revealed the common ultrastructural features of CRC of B-1558 pseudomonads: (1) thickened (as compared with vegetative cells) cell envelopes; (2) fine-granular cytoplasmic structure; and (3) condensed DNA in the nucleoid (Fig. 4). Apart from these features, the diversity of

Cell numbers obtained by direct count in an epifluorescent microscope after staining with Live/Dead reagent and the number of cultivated cells (by CFU)

Number	Culture of *P. aurantiaca* B-1558 (twofold N limitation, incubation time 6 months)	Culture of *P. aurantiaca* B-1558 (fivefold N limitation, incubation time 2 months)
Total cell number, ml^{-1}, among them	$(2.5 \pm 0.5) \times 10^8$ (100%)	$(2.1 \pm 0.4) \times 10^8$ (100%)
dead (red)	$(1.1 \pm 0.2) \times 10^8$ (44%)	$(0.91 \pm 0.07) \times 10^8$ (44%)
live (green)	$(1.4 \pm 0.3) \times 10^8$ (56%)	$(0.65 \pm 0.05) \times 10^8$ (31%)
"transitional" with yellow-green, yellow, or yellow-red fluorescence	Not revealed	$(0.52 \pm 0.05) \times 10^8$ (25%)
CFU number, ml^{-1}	$(3.0 \pm 0.4) \times 10^7$	1–2
Number of cells revealed by MPN, ml^{-1}	Not determined	1.1 (0.33–3.3) $\times 10^3$

ultrastructural organization of resting cells of pseudomonads was revealed. CRC from variant 1 (the medium with twofold N limitation, 6 months of incubation) (Fig. 4b) were characterized by an enlarged electron-dense periplasmic space up to 80–90 nm thick (in vegetative cells from exponential cultures, it is 40–50 nm thick; Fig. 4a), a more distinct and electron-dense peptidoglycan layer, and condensed chromatin, the compaction of which was especially pronounced in certain regions of the nucleoid. Anabiotic CRC from variant 2 (C_{12}-AHB, 1×10^{-4} M and 2.5×10^{-4}, 1.3 months) were characterized by a well distinguishable periplasmic space and the presence of electron-transparent inclusions, probably polyhydroxyalkanoates, in the cytoplasm (Fig. 4c, d). Two types of cells were revealed in CRC suspensions of variant 3 (incubation in $Na_2SiO_3 \cdot 9H_2O$ solution, 3 months). The first type (Fig. 4e) showed (1) the formation of loop-like "offshoots" (evaginates) from the outer cellular membrane, filled with an electron-transparent substance and giving rise to separate vesicles, and (2) the appearance of an external loose electron-transparent capsular layer encrusted with mineral particles from the outside. CRC of the other type occurring in variant 3 (Fig. 4f) were characterized by an enlarged electron-dense periplasmic space with fibrillar–granular structure and fine-granular texture of the cytoplasm, in which chromatin was poorly exhibited. Finally, rounded forms were revealed among the cells of cultures obtained in variant 5 (development on soil agar, 0.5–1 month) (Fig. 1d), differing from vegetative cells in external morphological characteristics (Fig. 1b). Particular features of these forms (Figs. 4g, 4h) are the presence of several (three to five) alternating layers of different electron density in the enlarged periplasmic space; the clumpy structure of the cytoplasm with electron-dense lumps; extensive intracytoplasmic electron-transparent inclusions similar in structure to the granules of poly-β-hydroxyalkanoates; and electron-dense clots of condensed DNA in the nucleoid. In some features of ultrathin structures, CRC on soil agar looked like azotobacter cysts, because they had layers similar to intine and exine, as well as inclusions of poly-β-hydroxyalkanoates, which occur extremely rarely in metabolically active cells.

DISCUSSION

Our findings make it possible to formulate two basic conclusions: (1) intensive formation of cystlike resting forms (CRC) in bacterial strains of the genus *Pseudomonas* requires specific conditions and (2) resting cells characterized by multilevel physiological, morphological, and ultrastructural diversity are formed in pseudomonad cultures under various growth conditions. Qualitative differences in the ability of *P. aurantiaca* B-1558 and *P. fluorescens* NCIMB 9046 CRCs to form colonies on standard media are the main indicator of their heterogeneity. CRC of pseudomonads in the variant of cultivation on the medium with fivefold nitrogen limitation were characterized by the loss of colony-forming ability after two months of incubation (Fig. 1), which agreed with the data on *P. fluorescens* cultures under nitrogen starvation [16]. Since CRC suspensions of this variant were shown to contain high numbers of cells recognized by the Live/Dead test as living and possessing undisturbed barrier membrane function, it seemed unlikely that cells not germinating on solid media were dead. Apparently, in this case we encountered either deeply dormant forms or transition of cells to a viable nonculturable state [3–5] associated by some authors with "vegetative dormancy" [17]. The emergence from the nonculturable state and recovery of proliferation ability require special procedures of resuscitation were described in the review [18]. The method based on introduction of sodium pyruvate into a solid

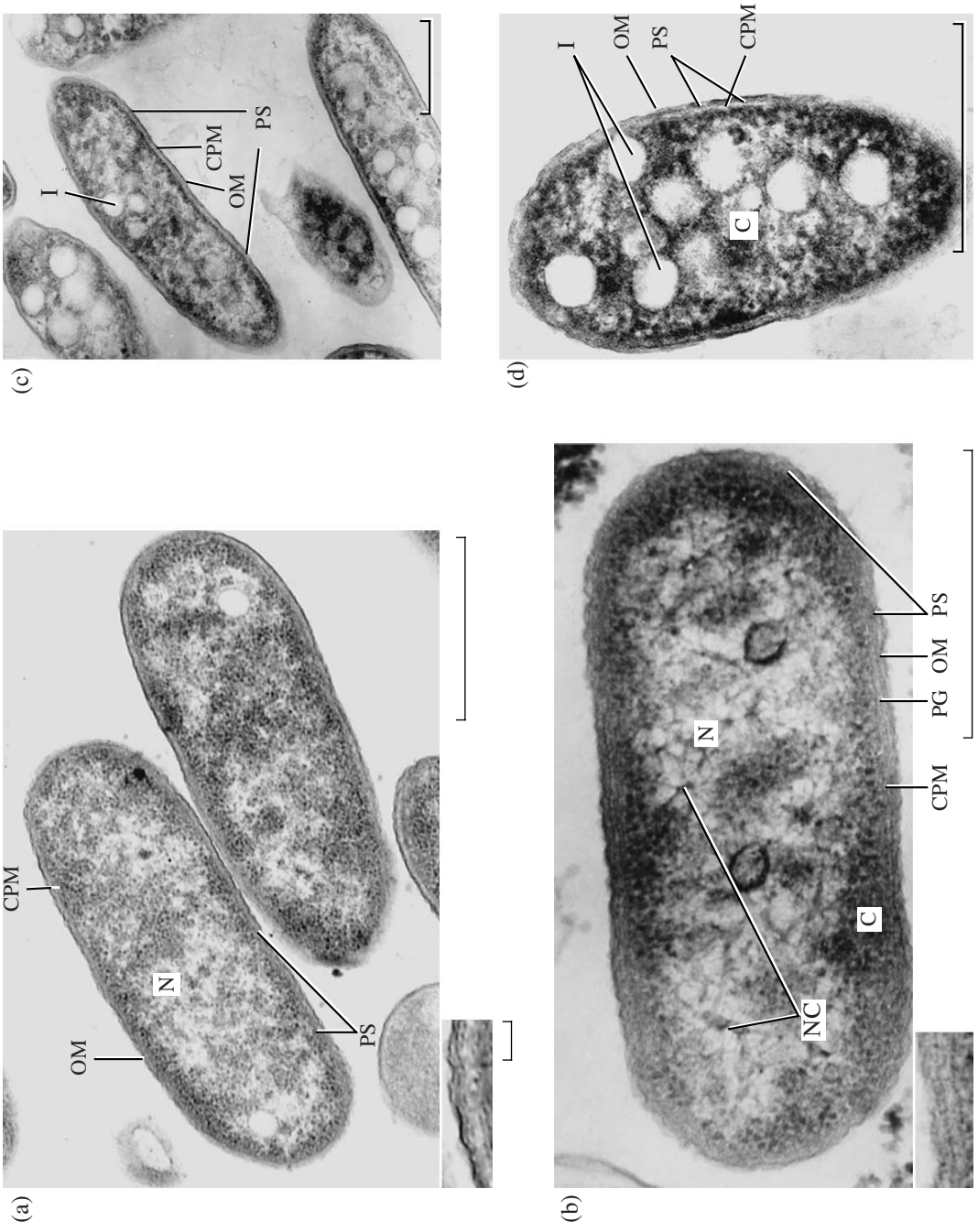

Fig. 4. Electron micrographs of the sections of *P. aurantiaca* B-1558 vegetative cells from the logarithmic growth phase (a) and the CRC obtained under different conditions: twofold N limitation, 6 months of incubation (b); introduction of C_{12}-AHB in concentrations 1×10^{-4} M (c) and 2.5×10^{-4} M (d), 1.3 months of incubation; incubation in the Na_2SiO_3 solution, 3 months (e, f); development on soil agar, 0.5–1 month of incubation (g, h). Designations: OM, outer membrane; PS, periplasmic space; PG, peptidoglycan; L, differentiated layers in the cell wall; CPM, cytoplasmic membrane; C, cytoplasm; N, nucleoid; NC, regions of nucleoid compaction (electron-dense lumps of DNA); I, inclusions; LP, loop-like protrusions; ECL, external capsular layer; P, mineral particles. Scale bar is 0.5 μm for the microphotographs of cells and 120 nm for cell wall fragments (a, b).

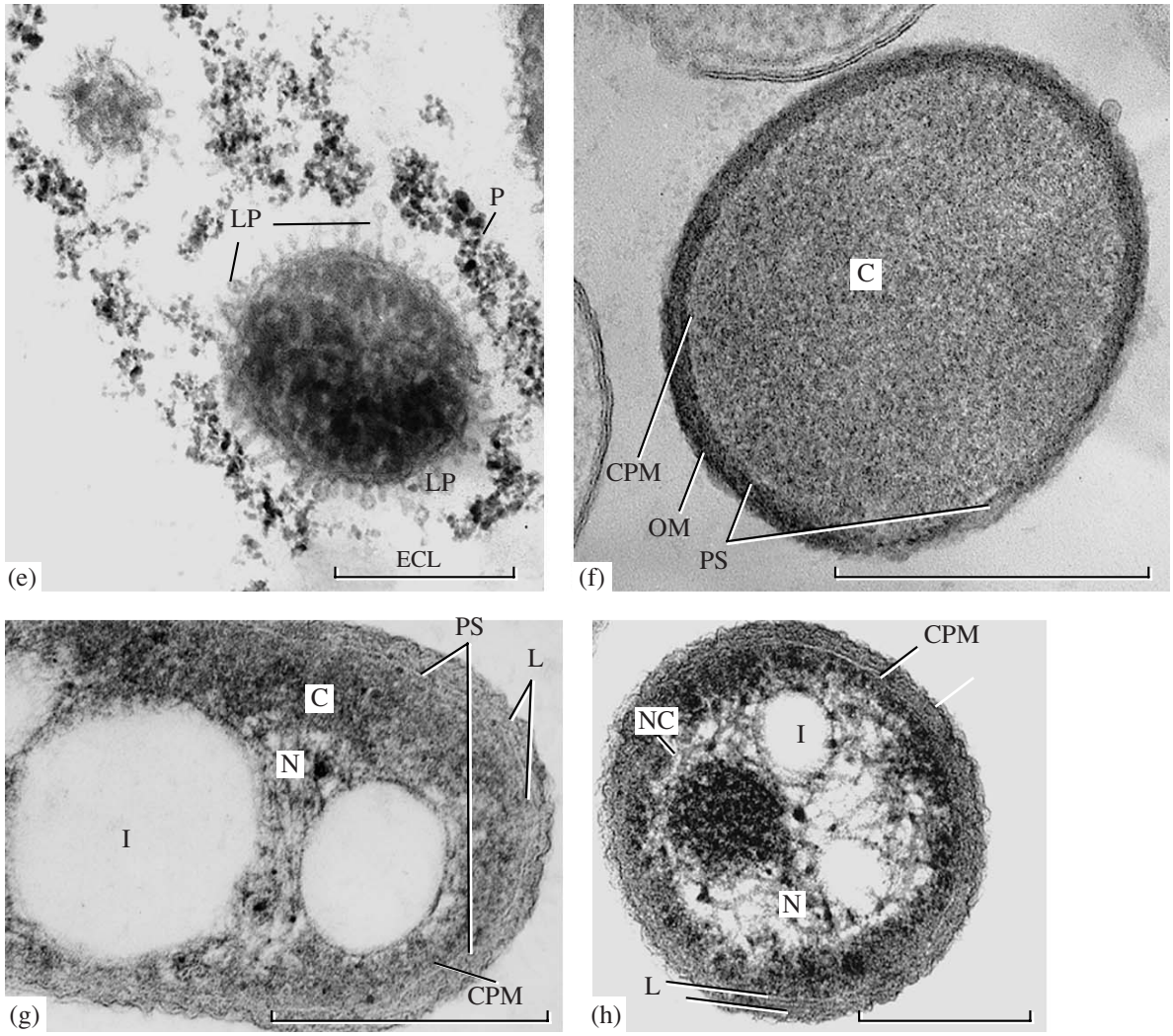

Fig. 4. Contd.

medium and providing a 100- to 10000-fold increase of CFU numbers of long-starved vibrio cells [12] proved to be inefficient in our experiments for recovery of the colony-forming ability of this variant of *P. aurantiaca* dormant forms. However, a minor increase in their quantity was obtained by cultivation in a liquid medium.

Other dynamics and the high level of preservation of the colony-forming ability (Fig. 1, Table) were typical of the CRC formed in six-month cultures in the medium under twofold nitrogen limitation; therefore no special reactivation procedures were needed. Thus, the level of nitrogen limitation in the medium is a significant factor for the formation of CRC with long-preserved viability in the cycle of development of microbial cultures.

The revealed dependence of preservation of the colony-forming ability in CRC on the concentration of introduced C_{12}-AHB (Fig. 3) was analogous to the one described previously [19]. This variant of obtaining pseudomonad CRC was based on the fact that autoregulatory factors d_1 that have the biological function of inducing the formation of anabiotic cystlike resting cells [9, 10] are represented in members of the genus *Pseudomonas* by alkyl hydroxybenzenes of the class of alkyl resorcinols [11]. The data on the dynamics of accumulation of factors d_1 (AHB) in developing *Pseudomonas* cultures [10] and low production of intra- and extracellular AHB by *P. aurantiaca* and *P. fluorescens* by [19, 20] suggested additional introduction of the analogues of these regulators (C_{12}-AHB) into the medium. Anabiotic CRC of this variant were interesting because, as they entered the new cycle of development, the ability of bacteria for phenotypic dissociation was displayed with particular distinctness. This will be a subject of our next publication.

It should be emphasized that the above-described methods of obtaining pseudomonad CRC simulate some important ecological conditions. Thus, cultivation in the medium with C > N unbalance can be con-

sidered as a model of starvation stress, while the increase of C_{12}-AHB concentration simulates a possible situation at drying up of some niches (in soils). At the same time, one should take into consideration the AHB biosynthesis ability not only in microorganisms by also in plants [21]. Variants 3 to 5 also take account of necessary simulation of natural conditions.

Thus, formation of the dormant forms of *P. aurantiaca* B-1558 and *P. fluorescens* NCIMB 9046 and their ability to resume growth without special resuscitation procedures are determined by cultivation conditions. Preservation of the proliferative potential in resting cells is influenced both by the properties of the organism itself and by a number of external factors. Thus, transition of starved *P. fluorescens* and *P. syringae* populations to the nonculturable state or retaining of the colony-forming ability depended, first, on the presence of the *lux* plasmids and, second, on storage temperature [4]. This conclusion is also confirmed by the data that the important conditions of survival of biomarker *P. fluorescens* strains introducers in the culturable or nonculturable state are pre-adaptation to starvation and soil properties (granulometric composition) [22].

When analyzing the data on the ability of pseudomonad CRC to sustain stresses, the differences in their resistance to heat treatment at 60°C (5 min) should be noted. In CRC of *P. aurantiaca* B-1558 obtained in variants 2 to 4 and incubated for 1.3–1.5 months, heat resistance was much higher than in vegetative cells (Fig. 3a). It should be emphasized that the dormant forms of other gram-negative bacteria (*Azotobacter* cysts) actually did not differ from vegetative cells in resistance to this dose of thermal exposure but sustained drying [23]. These parameters of stress resistance were typical of the dormant forms of *P. aurantiaca* B-1558 (Figs. 4g, 4h) obtained on soil agar (variant 5) and similar in ultrastructural peculiarities to azotobacter cysts.

Some other interesting facts are the revealed intrapopulation differences in resistance of pseudomonad CRC of variant 1 (the medium with twofold N limitation) to heating at 50, 55, and 60°C and the dependence of the population heterogeneity in thermal resistance on the duration of their storage (CRC age). The increase (by 5 orders of magnitude) of the portion of cells resistant to exposure at 60°C up to 1% in the course of CRC maturation (up to 6 months) indicates that the resting population contains highly stress-resistant forms, the number of which is determined not only by the conditions of their production but also by the periods of their formation in the dynamics of storage. The tendency for development in time of general stress resistance (to 9% ethanol, hydrogen peroxide, heating at 47°C, increase of NaCl content) has been shown previously for the cells of strain *P. fluorescens* R2f under conditions of carbon starvation (24 h) and for rifampicin-resistant mutant inoculated into soil (5–13 days) [22].

It should be particularly mentioned that introduction into the medium of sodium pyruvate, functioning as a scavenger of peroxide forms of oxygen [12], proved to be effective for resuscitation of some of the *P. aurantiaca* B-1558 CRC (variant 3) after heating (60°C, 5 min). This can be explained in the context of the fact that nearly all stress impacts (including heat shock) are associated with the damaging action of peroxide radicals [24]. This technique also revealed the heterogeneity of pseudomonad CRC of this variant by the need of pyruvate to recover colony-forming ability after heat treatment. It should also be noted that other, higher characteristics of heat resistance (93–95% of viability preservation after a week-long incubation at 62°C) were reported for *P. fluorescens* cells obtained under nitrogen starvation and germinating only after the special reactivation procedure [16]. The authors of the cited work took into consideration but did not specify the quantitative ratios of heat-resistant cells yielding macrocolonies at inoculations and microcolonies determined by direct count. Different methods of cell count (by CFU, mCFU, and with or without reactivation) are probably required to demonstrate the diversity of dormant forms in both growth resumption ability and heat resistance.

An important result is the finding of morphological diversity of pseudomonad resting cells (CRC) that possess certain common features (cell envelope thickening, modified structure of the cytoplasm, and compaction of the nucleoid) and specific peculiarities of ultrathin structure depending on the method of their production. These differences include the formation of an external capsular layer encrusted with mineral particles (variant 3), the size of the periplasmic space and the quality of the filling substance (variants 1 to 3), the presence of easily distinguishable layers in the cell wall (variant 5), and the presence of abundant inclusions (variants 2 and 5). The CRC of *P. aurantiaca* B-1558 described in this work for variants 1 and 3 and in work [13] are similar in their ultrastructural characteristics to the cyst-like forms of gram-negative bacteria *Legionella pneumophila* [25], while CRC formed on soil agar (variant 5) are similar to the cysts of *Azotobacter* and *Azospirillum* [26, 27]. Formation of morphologically different types of dormant pseudomonad forms from more simply organized vegetative cells is evidence of their structural plasticity and a realization of the cytodifferentiation programs, which may be considered an important adaptation mechanism.

Based on the array of described characteristics, pseudomonad CRCs have been categorized as dormant bacterial forms in accordance with the criteria accepted in sporology [15]. However, interpretation of the term "dormancy" for non-spore-forming bacteria in the literature is ambiguous, too broad, and encompasses cells of different physiological states. This term covers viable nonculturable cells that require special procedures for resuscitation [17, 18], as well as nonculturable cells that do not respond to the introduction of nutrients [28],

including inactive ones (resting cells of *P. fluorescens* CHA0 [5]; and the cells of *P. aeruginosa* from biofilms with undetectable alkaline phosphatase activity, which are considered to be absolutely dormant [29, 30]). These different, overlapping but not identical states of proliferative (absence of division) and metabolic dormancy, as well as the multilevel biodiversity of pseudomonad CRC that we have shown, provide for various ecological functions. It seems that the long-term survival of gram-negative non-spore-forming bacteria is achieved due to the formation of cystoid dormant forms characterized for pseudomonads in the present research and found in soil and the ground [7, 8, 13, 31].

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