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EXPERIMENTAL ARTICLES

Heterogeneity of the Populations of Marine Luminescent Bacteria *Photobacterium leiognathi* under Different Conditions of Cultivation

S. E. Medvedeva[1], O. A. Mogil'naya, and L. Yu. Popova

Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Akademgorodok, 50, Krasnoyarsk, 660036 Russia

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Abstract—Manifestation of pleiotropic effects in the isogenic variants of the luminescent bacterium *Photobacterium leiognathi* 54 was investigated. The decrease or increase of the expression level of bioluminescence was caused by changes in *lux* operon regulation. The dynamics of the bioluminescence of dark and dim variants did not differ from the dynamics of the initial luminescent variant, but dependence of the level of luminescence intensity on the exogenous autoinducer of the *lux* operon was revealed. The investigated variants of *P. leiognathi* 54 inherited fairly stable morphological characteristics, colony architectonics, level of luminescence, and activity of some enzymes; variants with reduced bioluminescence formed colonies of the S type. Stable bright variants with S- and R-type colonies appeared both in the initial strain population and in the dark variant population, but with smaller frequency. Populations of the bright variant with R-type colonies were most heterogeneous; this can be determined by the lack of glucose repression of the bioluminescence in contrast to other investigated inherited variants of *P. leiognathi*.

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Key words: bioluminescence, luminescent bacteria, morphology of colonies.

The fundamental dialectic paradigm of evolution (stability vs. variability) lies at the intersection of molecular genetics, population genetics, and ecology [1, 2]. Population variability in the phenotypic manifestation of gene expression is characteristic of the majority of microorganisms [3, 4]. The expression of many genes can alter with changes in the substrate [5]; this is particularly important for the maintenance of collections of microorganisms with specific characteristics. The heterogeneity of microbial populations is caused by a wide variety of bacteriophages and also by migratory genetic elements, transposons, integrons, and plasmids [6, 7]. Adaptive mutations and regulatory mechanisms play a special role in the manifestation of heterogeneity: they cause cumulative effects in the phenotypic variability of microbial populations [2, 8, 9]. Such cumulative effects caused by the common regulation of several operons are known for marine luminescent bacteria [10]. For instance, coordinated regulation of the genes of riboflavin synthesis and of *lux* genes occurs in the cells of *Photobacterium phosphoreum* [11]. Genetic polymorphism was revealed in symbiotic luminescent bacteria *P. leiognathi* [12] and in the luminescent bacteria of the genus *Vibrio* [13]. With the change of their habitat, luminescent bacteria lose their usual physicochemical environment and nutrient sources [14]. Hence, the metabolic activity of the cells changes, which can result in decrease or increase of the bioluminescent signal.

The collection of cultures of marine luminescent bacteria (CCIBSO 836), isolated from different parts of the World Ocean, has been maintained for many years in the Institute of Biophysics, Siberian Division, Russian Academy of Sciences (IBSB). During re-plating and cultivation, dark, dim, and bright variants with different intensity of bioluminescence appear in the populations of luminescent bacteria [15]. The heterogeneity of populations of luminescent bacteria is primarily assessed as the visual heterogeneity of luminescence of the colonies and colony morphology on plates with solid nutrient medium. The application of electron microscopy to investigate morphologic changes makes it possible to assess in detail the structure of the population, the processes of cell differentiation, and dissociation into subpopulations. Such information is particularly important for the understanding of the reasons for the appearance of variants with reduced activity of the bioluminescent system in the initial culture of luminescent bacteria. Furthermore, knowledge of the characteristics of population heterogeneity, particularly, of the cytomorphological characteristics, is necessary for complete description of the available strains of lumi-

[1] Corresponding author; e–mail: ccibso@ibp.ru

nescent bacteria, in order to fill the databank of this microbial group maintained in the IBSB Collection of cultures (CCIBSO, http://bl.ibp.ru) [16].

In the present work, the results of investigation of the heterogeneity of population of luminescent bacteria *P. leiognathi* 54 depending on the conditions of cultivation and the functional activity of luciferase-producing strains are discussed.

MATERIALS AND METHODS

Luminescent mesophilic bacterium *Photobacterium leiognathi* 54 was obtained from the IBSB collection of cultures; the isogenic variants of the strain *P. leiognathi* 54 were isolated by passages on different media.

In the present work, the synthetic medium (M) was used, containing (g/l): Na_2HPO_4, 6; KH_2PO_4, 3; $(NH_4)_2SO_4$, 2; NaCl, 3.0. After autoclaving, 1 ml of 20% $MgSO_4$ solution, 1 ml of 0.5% $CaCl_2$ solution and 10 ml of 20% glycerol or glucose solution were added aseptically. The medium was supplemented with 5 g/l of peptone (P) and fish extract (FE) in the ratio 1 : 1 with total medium to enrich it with nutrients. The solid media contained agar (22 g/l).

Batch cultivation of the strains was performed in test tubes or in Erlenmeyer flasks on a temperature-controlled shaker at 28°C. The optical density of the cell suspension was measured with a KFK-2MP photocolorimeter (Optical and Mechanical Plant, Zelenograd) in a 0.5 cm quartz cuvette at 540 nm (green filter). The error of the optical density measurements did not exceed 11%. The luminescence intensity was measured with a bioluminometer designed in Nauka SKTB (Krasnoyarsk). The signal from the luminometer was recorded by a M-95 microampermeter. This setup allows measurements of the luminescence intensity within the range from 10^{-4} to 10^2 μA (1 μA = 4×10^9 quanta/s). The error of the luminescence measurement did not exceed ±5%. Statistical data processing was performed with Microsoft Excel 7.0 software package.

The coordination of the synthesis of luciferase and of aldehyde- producing enzymes was assayed indirectly by adding to the bacterial suspension 0.2 ml/ml of 0.15% alcohol solution of myristic aldehyde (Merck, Germany). The increase in intensity upon addition of the aldehyde indicated that the exogenous aldehyde compensated for the deficiency of the cellular aldehydes; the aldehyde synthesis in this cell variant was therefore insufficient [17].

Variants of *P. leiognathi* 54 were grown in complete liquid medium in batch mode to determine the changes in the dynamics of luminescence development; they were then compared with the dynamics of the wild type strain under such conditions. To determine the changes in the synthesis of *lux* operon autoinducer in the variants, the wild type strain was grown in batch mode up to the middle of the phase of luminescence intensity increase; the cells were then precipitated by centrifugation. The supernatant was sterilized by autoclaving at 0.5 atm and was added in equimolar quantities to the nutrient medium [18].

For electron microscopic investigations of the colony structure, the method of colony fixation in situ was applied. The cell structure and the colony architectonics were investigated on panoramic (with a large area) ultrathin sections using a JEM-100C electron microscope [19].

RESULTS AND DISCUSSION

Heterogeneity of the manifestation of bioluminescence in luminescent bacteria. The dynamics of the manifestation of bioluminescence in luminescent bacteria is rather variable depending on their growth conditions. In batch mode of cultivation, the intensity of cell luminescence changes in parallel with increasing quantity of enzymes and substrates participating in the reaction of bioluminescence. The concentration of the synthesized enzymes and substrates depends on a number of regulatory mechanisms which involve the protein repressor of *lux* operon and the corresponding inducer of the homoserine lactone class. It also depends on the complex of the catabolism activating protein (CAP) with cAMP, which ensures the positive regulation of the action of bacterial operons. The inhibition of the bioluminescence in media with glucose occurs due to the last mechanism; the repression of the *lux* operon in minimal medium is rapid (Fig. 1). In minimal media, the synthesis of regulatory proteins is limited due to lack of nutrients, and the specific intensity of luminescence per cell is always higher than in rich media at high growth rates. In media with peptone and, particularly, with fish extract, the dynamics of luminescence depends on the phase of bacterial growth to a greater extent than in minimal media. The relationship between the bioluminescence and various metabolic processes in the cell explains such differences in the manifestation of the dynamics of luminescence intensity depending on the media composition (Fig. 2). Thus, changes in *lux* operon expression may depend not only on mutations in its genes or on the medium composition but on the changes of regulator control that can result in both increase and decrease in the synthesis of the enzymes responsible for the bioluminescence reaction [17]. If the regulation is common to the cells, heterogeneity in the populations of luminescent bacteria may be pleiotropic.

K variants (spontaneously arising variants with decreased bioluminescence) do not entirely lose their ability to emit light; this is evidence of changes in the regulation of *lux* operon expression. These variants reveal weak light emission caused by the low content of luciferase and of all or several substrates of the luciferase system (Fig. 3). K variants are rare in the cultures maintained under conditions of fast growth and intense aeration, while limited oxygen supply in dense

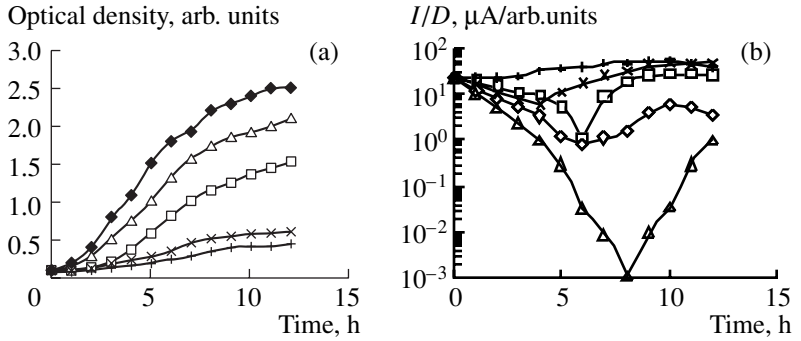

Fig. 1. The dynamics of the growth (a) and the luminescence (b) of luminescent bacteria *P. leiognathi* 54 in batch culture on different media: with glucose (×, Mglu), glycerol (|, Mglyc), glucose and peptone (△, Pglu), glycerol and peptone (□, Pglyc), peptone, and glycerol and fish extract (◆, PglycFE).

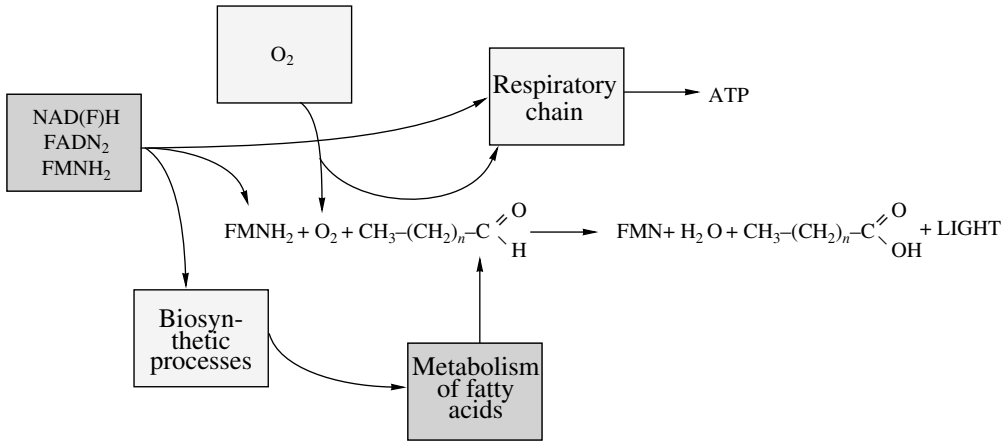

Fig. 2. The scheme of the reaction of bioluminescence and the related reactions of substrates and inducers synthesis.

cultures and high temperature are favorable for the emergence of variant forms. However, the reasons for the origin of this phenomenon, which is common among luminescent bacteria, are still obscure. For instance, the bright luminescent variant of the marine strain *P. leiognathi* 54 was maintained on the standard medium with peptone and preserved a high level of luminescence for many years, but exhibited practically no population heterogeneity in this respect. However, rapid accumulation of dark and dim variants in the population of *P. leiognathi* 54 occurred after passages on rich media (supplemented with fish extract) or after storage under petrolatum in semiliquid agar (oxygen limitation) (Fig. 3, phenotypes 2, 3). On the contrary, cultivation of *P. leiognathi* 54 in poor medium without peptone resulted in the emergence of variants with constitutive expression of *lux* operon in the population (Fig. 3, phenotypes 4, 5). Variants with intermediate bioluminescence intensity were also isolated.

Investigation of the bioluminescence dynamics of dark, dim, and bright variants in batch culture on media of different composition revealed the regulatory character of such manifestations of population heterogeneity, determined by the metabolic activity of the cells. The data presented on Figs. 1, 3 demonstrate that the bioluminescence of the initial phenotype of the strain *P. leiognathi* 54 (phenotype 1) depended on the presence of glucose in the medium (catabolite repression of the *lux* operon). Under these conditions, the bioluminescence level did not depend on the addition of an exogenous substrate (C_{14} aldehyde) to the cell suspension. The addition of the supernatant of a bright cell culture with the autoinducer for the *lux* operon reduced the duration of the latent period in the bioluminescence dynamics in phenotypes 1–3 (Fig. 3, curve *3*) in comparison with the control (Fig. 3, curve *1*).

The cells with low bioluminescence level that appear in the population of *P. leiognathi* 54 preserve their basic parameters in the bioluminescence dynamic, although they are more dependent on additional autoinducer (phenotype 2) or on exogenous myristic aldehyde (phenotype 3). The catabolic control may be impaired in the cells of certain dark variants (for instance, in the variant with phenotype 3, Fig. 3). Impairing of the catabolic control is more pronounced in the cells with constitutive expression of the *lux* operon (phenotype 4 and

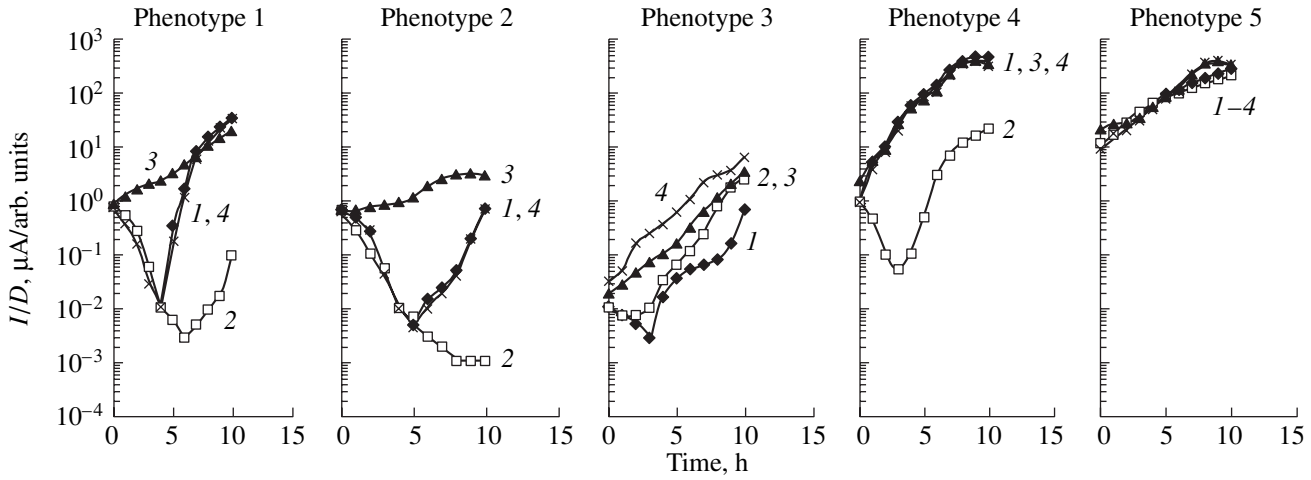

Fig. 3. Specific luminescence of isogenic variants emerging in the population of luminescent bacteria *P. leiognathi* 54 during long-term storage. Phenotype 1, $Rep^+Ind^+Cap^+Ald^+$ (original wild type phenotype); phenotype 2, $Rep^{++}Ind^-Cap^{++}Ald^+$ (the variant with amplified control of *lux* genes expression); phenotype 3, $Rep^+Ind^+Cap^-Ald^-$ (the variant with impaired catabolite control of *lux* genes expression and with bioluminescence depending on exogenous aldehyde); phenotype 4, $Rep^-Ind^{++}Cap^+Ald^+$ (the variant with impaired control of *lux* genes expression visible in constitutive synthesis of the *lux* operon enzymes, with but conserved catabolite control of bioluminescence); phenotype 5, $Rep^-Ind^{++}Cap^-Ald^+$ (the variant with completely impaired control of *lux* genes expression, constitutive synthesis of the *lux* operon enzymes in the absence of catabolite control of bioluminescence). *1*, specific luminescence on the medium with peptone and glycerol; *2*, specific luminescence on the medium with peptone and glucose; *3*, specific luminescence on the medium with peptone, glycerol, and additional inducer; *4*, specific luminescence on the medium with peptone and glycerol after addition of exogenous myristic aldehyde.

phenotype 5). However, the dependence of the bioluminescence dynamics on the additional autoinducer or on exogenous aldehyde was not detected in the cells with such an unregulated phenotype of *lux* operon expression.

The knowledge of the tendency of formation of dark variants in luminescent bacteria populations and of the factors that determine the reversible transformation between bright and dark forms is necessary for understanding the ecology of luminescent bacteria. More profound investigations of the properties of the variants with changed luminescence expression can extend the idea of the role of the luminescent system in the metabolism of luminescent bacteria and determine the relationship between growth and bioluminescence. Such relationships in luminescent bacteria grown on solid nutrient medium can be determined visually in a dark box.

Structure of colonies and cells. The investigation of the dynamics of inherited variability of *P. leiognathi* 54 revealed several inherited variants that appeared with the frequency 10^{-3}–10^{-5}, having different luminescence intensity (Fig. 4) and physiological and biochemical properties [15]. All the selected variants exhibited fairly stable inheritance of the investigated morphological characteristics of colonies, the luminescence level, and the activity of *lux*-operon enzymes. Three-day colonies of the investigated variants of the luminescent bacteria *P. leiognathi* 54 had distinct morphological characteristics. They differed in their surface geometry, density, color, and luminescence intensity (table). Electron microscopy of the structure of three-day colonies revealed that they consisted of cells of different ultrastructure and with different localization within the colony (Figs. 5–7).

On the basis of the revealed difference, the observed morphological features were classified in accordance with the cytoplasm condition and the presence and character of the inclusions. The first type of cells was

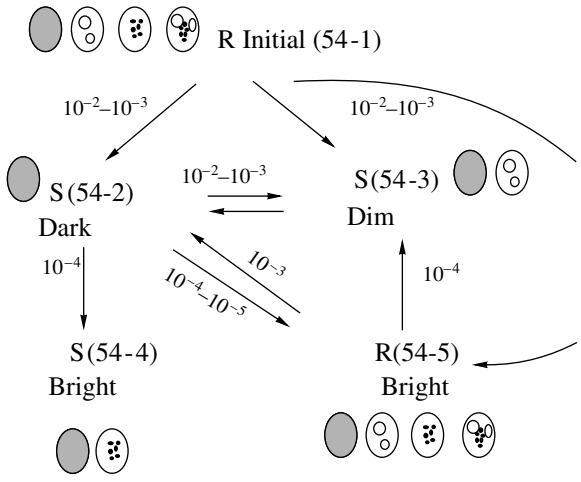

Fig. 4. Scheme of the mutual conversions of isogenic variants in the population of *P. leiognathi* 54. The morphologic types of colonies (R, S), ultrastructural differences of cells in these colonies (see more detailed descriptions in text) and frequency of the variants' mutual conversions are shown.

Morphological features and cell composition of the colonies of variants arising in *P. leiognathi* 54

Variants	Morphological features of colonies	Number of cell types in colony		
		1-day colonies	3-day colonies	7-day colonies
54-1	Rough, semitransparent, whitish, luminescent	5 types	5 types	5 types
54-2	Smooth, clear, yellowy, dark	1 type	2 types	2 types
54-3	Smooth, dense, whitish, dim	1 type	3 types	4 types
54-4	Smooth, dense, whitish, bright	2 types	4 types	2 types
54-5	Rough, semitransparent, whitish, very bright	5 types	7 types	6 types

coccobacilli; the outer membrane of the cell wall has a slightly wavy profile, the cytoplasm is homogeneous and filled with ribosomes, and the thin fibrillar nucleoid is located in the central zone (Fig. 5a). The cells of the other types can be considered derivatives of the first cell type. The second type is characterized by the presence of electron-transparent inclusions of poly-β-hydroxybutyric acid in the cytoplasm (Fig. 5b); in the nucleoid

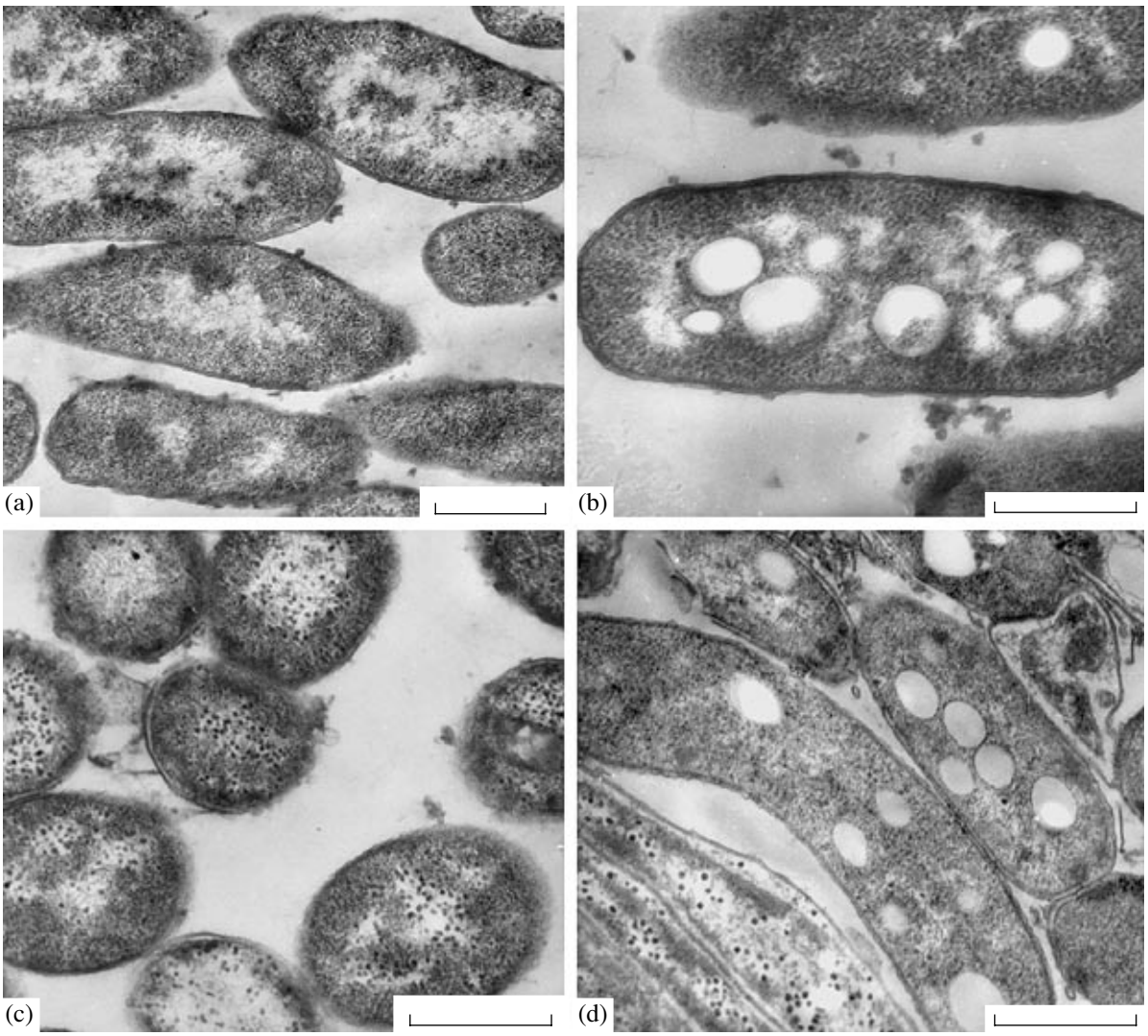

Fig. 5. Ultrastructure of the cells from 3-day colonies of isogenic variants of *P. leiognathi* 54: a, cells in smooth (S) colonies of dark variant 54-2 (phenotype 2); b, cells with electron-transparent inclusions in smooth (S) colonies of dim variant 54-3 (phenotype 3); c, homogeneous population of cells with electron-dense granules in smooth (S) colonies of luminescent variant 54-4 (phenotype 4); d, heterogeneous population in rough (R) colonies of bright variant 54-5 (phenotype 5). Scale bar, 0.5 μm.

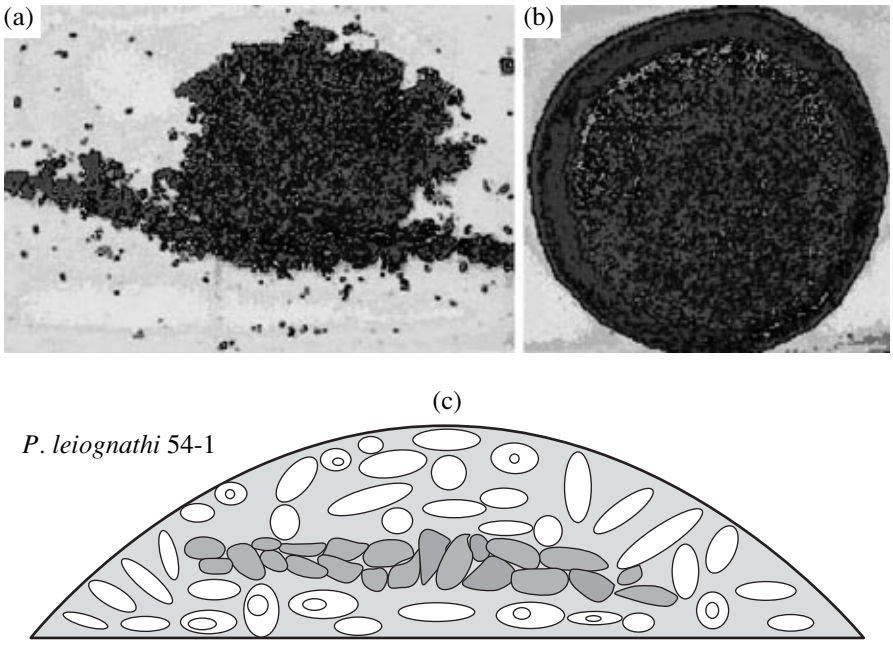

Fig. 6. Microcolony formation (a) and mature colony (b) of *P. leiognathi* 54, and schematic presentation of the location of active (light) and lysed (dark) cells in colonies of *P. leiognathi* variants (c).

zone of the cells of the third type, electron-dense granules with diameter up to 30 nm are present (Fig 5c); in the fourth type, both types inclusions are present (Fig. 5d). Degrading cells with the ultrastructure corresponding to the above four types but with a different cytoplasm condition also occurred. Thus, 8 types of cells resulting from the different combination of characteristics were identified. It turned out that the colonies of each variant contained a selection of these 8 morphological types. Each type of colony is characterized by its typical set (table). A distinct correlation between the cell types of the colony and its morphological characteristics was revealed. The following pattern can be noted upon the analysis of the cytomorphological characteristics of the isogenic variants of *P. leiognathi* 54, of the process of colonies formation, and of their physiological and biochemical characteristics and mutual transformations. The bright luminescent variant 54-5 (Fig. 3), forming rough colonies, has stable luminescence, but its cellular composition is extremely heterogeneous (Table, Fig. 5d). Dark variant 54-2 (table, Figs. 3, 5a), with smooth colonies, has a homogenous morphological type of cells but unstable luminescence because clones with different luminescence level (i.e. other variants) are most prone to appear in its population (Fig. 4).

The colonies of the dark variant 54-2 *P. leiognathi* consist of one type of cells with homogeneous cytoplasm without inclusions (Fig. 5a). Importantly, this bacterial type along with others occurred in all isolated variants. Furthermore, only such cells are present in young colonies (18 h of growth) of bright luminescent variants 54 and 54-5 with the most heterogeneous composition (initial phenotype 1 and phenotype 5, respectively; Fig. 3) when the colonies can already be visually detected but do not yet glow. Liquid batch culture at the beginning of the exponential phase of growth also consists of cells of the first type. This type of cell is probably the original one, and other types differentiate during colony growth (Fig. 4). The descriptions of the colonies of freshly isolated luminescent bacteria indicate that the dark S variant is probably the original one under environmental conditions and prevails there. The dark S variant composed of "undifferentiated" cells is probably important for the preservation and stabilization of the species in natural conditions. The R variants with high bioluminescence level and a more varied cell composition are probably important for the adaptation of the species to different environmental conditions.

Electron microscopy of the colonies of the investigated variants of *P. leiognathi* 54 revealed that their architectonics corresponded to classical representations (Fig. 6). The vertical section of colonies demonstrated the basic tendency characteristic of the cells of all variants: the actively operational organisms formed layers located at the periphery of the colony, while degrading cells were located in the central part of the colony in the form of a local layer that could reach a third or more of the colony height (Fig. 7). The cell layer closest to the surface of nutrient agar occupied, usually, a lesser volume than the same cells from the outer portion of the colony. In the lysis zone, the cells were impacted, whereas at the periphery they were located fairly loosely although maintaining contact with each other. A zone of lysis cells was shown to

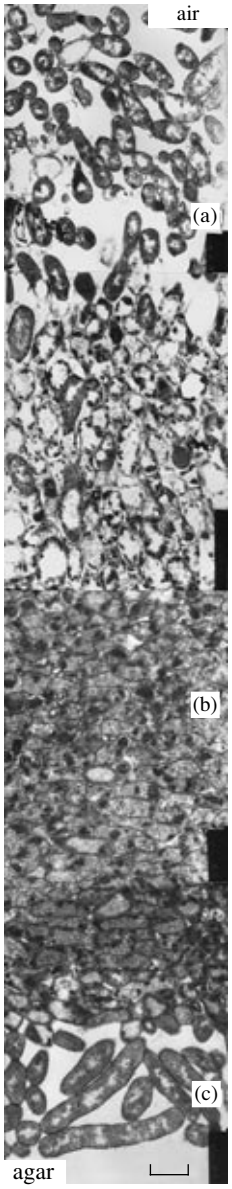

Fig.7. Panorama of the section of a colony of the initial variant of *P. leiognathi* 54-1: a, layer of active cells adjacent to air; b, lysis zone; c, layer of cells adjacent to agar. Scale bar, 1 μm.

occur in the colonies of *P. leiognathi* 54 as early as a day after transfer.

The investigated variants of photobacteria differed in many characteristics affecting the main features of intracellular metabolism. In particular, the change in the fatty acid composition of membranes, namely, the higher unsaturation coefficient of the smooth variants, led to a change in membrane permeability and decreased sensitivity to antibiotics and other toxic substances [15, 20]. Thus, the ability of cells to form subpopulations with reduced expression of *lux* genes but more resistant to environmental toxic compounds is of ecological importance for the survival of populations of luminescent bacteria. The existence of stable variants, both bright ones (the variant 54-4) and dark ones (54-2), which are not capable of conversion to other isogenic forms (or convert with low frequency), may indicate their adaptation to the habitat. The variety of hereditary variants indicates the existence of several regulators. The presence or absence of these regulators leads to significant reconstruction of cell metabolism and, accordingly, to the emergence of certain cell clones and the formation of different types of colonies. The bright variant with homogeneous cell composition, which forms colonies of smooth type, exhibits the constitutive type of bioluminescence dynamics and retains catabolite regulation by glucose; it is the most preferable for long-term storage in collections.

Glucose repression of biofilm formation was observed in all the variants with glucose repression of bioluminescence. Preliminary results concerning comparison of optimal conditions for formation of structured communities and for luminescence revealed a number of regularities. The variants of *P. leiognathi* 54 formed biofilms at a low concentration of NaCl (0.1%), although visible bioluminescence was detected only at higher concentrations of NaCl (3–5%). The discovered characteristic of the formation of communities of marine luminescent bacteria associated with phase boundaries may be used for determination of their preferable habitats in marine ecosystems.

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