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EXPERIMENTAL ARTICLES

# ATP Pool and Bioluminescence in Psychrophilic Bacteria *Photobacterium phosphoreum*

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**Abstract**—Bioluminescence activity and ATP pool were investigated in the cells of psychrophilic bacteria *Photobacterium phosphoreum* collected from the exponential and stationary growth phases and immobilized in polyvinyl alcohol (PVA) cryogel. In liquid culture, ATP pool remained at an almost constant level throughout the luminescence cycle (over 100 h). The ATP pool in the stationary-phase and PVA-immobilized cells remained constant throughout their incubation in the medium (over 200 h) and in 3% NaCl solution (over 100 h). Quantitative assessment of integral photon yield and ATP pool indicated that bioluminescence decay in growing or stationary cells was not caused by limitation from the energy substrates of the luciferase reaction. Kinetic and quantitative parameters of emission activity and ATP pool excluded the possibility of formation of the aldehyde substrate for luciferase via reduction of the relevant fatty acids in NADPH and ATP-dependent reductase reaction and its oxidation in the monooxygenase reaction. Our results indicate that the aliphatic aldehyde is not utilized in the process of light emission.

*Keywords*: psychrophilic photobacteria, luminescence cycle, ATP pool, cell immobilization, polyvinyl alcohol
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The luminescence activity of photobacteria is controlled by a complex of factors, primarily by the substrates and cofactors of the luminescence system, the autoinducers, temperature, and pH. This is reflected in the profile of the luminescence activity of a growing culture and in the luminescence decay kinetics in the stationary-phase cells [1–3]. In a growing culture, duration of the luminescence cycle, which includes the phases of induction, stationary luminescence, and decay, varies in different species. In *Vibrio harveyi*, the whole cycle of luminescence takes 12–14 h and occurs during the logarithmic growth phase [4, 5], while duration of the luminescence cycle in *Photobacterium phosphoreum* is 1 to 7 days [6, 7].

The processes that control the transition to the decay stage have not yet been well understood. Luminescence decay may be due to changes in the cultivation medium (pH shift or accumulation of inhibitory products), alterations in cell metabolism (biosynthesis of alternative electron acceptors, uncoupling of the electron transfer chain, or an increase in non-luminescent leakage in the course of electron transfer to luciferase), substrate limitation, or luciferase inactivation.

Of particular importance for the emission process is the ATP pool, an integral measure of both light and dark reactions. Various authors provided the values for intracellular ATP content in photobacteria from 0.2 to $2.0 \times 10^{-18}$ mol per cell [4, 8]. Although ATP is not directly involved in the light reaction, the oxidation of the reduced flavin and of the aldehyde molecule by bacterial luciferase with an emission activity of $10^4$–$10^5$ quanta $\times$ $s^{-1}$ $cell^{-1}$ may potentially compete with ATP synthesis for electrons.

The data on the dynamics of the ATP pool during the growth of a photobacterial culture are contradictory. Ulitzur and Hastings [4] revealed a 10–20-fold decrease in ATP pool during luminescence induction in submerged *Vibrio* (*Beneckea*) *harveyi* culture. Discrepant data on the dynamics of the ATP pool in a growing culture were obtained by Karl and Nealson [8], who analyzed the ATP and GTP pool, oxygen uptake, and the adenylate system load in four photobacterial species. They established that all the energy-related parameters remained constant during the growth cycle, whereas luminescence varied by several orders of magnitude. The authors cited discussed the contradictions between their data and the results reported in [4] and suggested that the decrease in ATP pool in *V. harveyi* was probably due to ATPase activation. Hence, the relationships among the bioenergetic processes that control both luminescence induction and decay in a growing bacterial culture have not been elucidated up to now. To some extent, this is due to a short period of the luminescence cycle in the photo-

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bacterial species used in the works cited. Psychrophilic strains of the bacterium *P. phosphoreum* are characterized by the longest luminescence period (over 100 h) in submerged culture [7]. Immobilization of bacterial cells (in contrast to free cells) makes it possible to prolong their luminescence [9, 10], which is a prerequisite for a more adequate assessment of their luminescent and bioenergetic characteristics. The cryogel of polyvinyl alcohol (PVA) is widely used to immobilize the cells of diverse groups of microorganisms [11]. The data on the immobilization of environmental and genetically modified strains with cloned bacterial luciferase genes are presented in [9, 11, 12]. A detailed study concerning the luminescence-stabilizing factors and emission-related and kinetic parameters of PVA-immobilized *P. phosphoreum, Vibrio fischeri*, and *V. harveyi* was conducted by us earlier [9, 13]. Cryogenic immobilization of photobacteria in PVA cryogel made it possible to considerably increase the duration and stability of light emission. If the preparations were incubated at 4°C, a stable emission level persisted for 200 h and a detectable emission level of immobilized cells was maintained for up to one month.

The goal of the present work was to investigate the dynamics of the ATP pool and luminescence activity in a growing culture, stationary phase, and immobilized cells of the psychrophilic bacterium *Photobacterium phosphoreum*.

## MATERIALS AND METHODS

**Bacterial strain and cultivation conditions.** These studies were conducted with the luminescent bacterium *Photobacterium phosphoreum*, strain 331 (KKM Moscow State University), which was isolated from the intestines of the fish *Myoxocephalus scorpius* in the White Sea.

The bacterium was grown in submerged culture in a complex medium containing the following (g/L): NaCl, 30.0; $Na_2HPO_4$, 5.3; $KH_2PO_4 \cdot 2H_2O$, 2.1; $(NH_4)HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; yeast extract, 1.0; peptone, 5.0; and glycerol, 3.0; pH 7.5 [7] at 20°C for 22 h (until the late logarithmic growth phase) to attain a biomass density of $5–8 \times 10^9$ cells/mL.

**Biomass harvesting.** The culture (500 mL) was centrifuged (3000 rpm, 10 min), washed once with Na-phosphate buffer with 2% NaCl, pH 7.6, and recentrifuged under the same conditions, whereupon the raw biomass pellet was used in the immobilization procedure.

**Immobilization**. The cells of photobacteria were immobilized according to [9]. A homogeneous biomass suspension in 13% solution of PVA (molecular mass 48000) was prepared. The PVA solution was based on the cultivation medium. Cell suspension (200 μL) was dispensed into the wells of a 96-well plate, frozen at –20°C, incubated in the frozen state for 17 h to form the cryogel, and stored at –8°C. The preparation obtained contained the following (wt %): bacterial biomass, 0.62 and polyvinyl alcohol, 6.8. The plates with the granules were thawed for 17 h, and the thawed granules were stored at 4°C in 0.1 M Na-phosphate buffer with 2% NaCl, pH 7.6.

**Determination of emission activity.** The bioluminescence of cell cultures, suspensions, and immobilized cells was assessed on an 1250 LKB–Wallace luminometer and expressed in relative units (r.u.). Absolute quantum yield values (Q, quanta/s) were determined according to the standard suggested by Hastings and Weber [15] : 1 r.u. = $2 \times 10^9$ quanta/s. The bioluminescence activity of a cell cultures or suspensions was assessed at concentrations of $10^6–10^4$ cells/mL in 0.1 M Na-phosphate buffer with 2% NaCl, pH 7.6. The activity of immobilized preparations was determined as the maximum luminescence of one granule in 1 mL of the same buffer after equilibrating the temperatures of the granules and the solution for 1–2 min; the values were normalized by dividing them by the cell number in the sample.

**Biomass measurement.** Cell concentrations in a growing culture or suspension were determined on a Beckman 35 spectrophotometer from the optical density value ($A_{630\ nm}$, $l = 1$ cm), using the relevant calibration curve. Quantitative assessment of the cell content in the granules was based on determining ATP by the bioluminescence method using firefly luciferase [17]. In all tested systems, the bioluminescent agent produced by Lumtek (Russia) was used.

**ATP determination.** Cell disintegration and ATP extraction were carried out by treating cell suspensions or immobilized cells-containing PVA preparations with DMSO: 100 μL of a bacterial suspension or one cryogel granule with immobilized cells were added to 1 mL of DMSO. Thereupon, bioluminescent assessment of ATP content in the samples was performed after 10 min of incubation. The measurement system was as follows: 1 mL of 0.1 M Tris-HCl buffer, pH 7.6 + 20 μL of the sample + 50 μL of the luminescent agent with firefly luciferase. Maximum emission intensity served as the quantitative criterion. ATP concentration was determined using the emission intensity $(I - I_{background})$/[ATP] calibration plot.

The results were statistically processed using the Excel software. Each value plotted in a graph is the mean value based on three repeats of an experiment.

## RESULTS

Fig.1 contains the data on growth dynamics, specific bioluminescence activity, and ATP pool of a submerged culture at 20°C. It was revealed that the time course of bioluminescence activity and that of the ATP pool were asynchronous. Duration of the luminescence cycle was over 100 h. The ATP pool decreased insignificantly during the logarithmic phase and was maintained at a constant level (0.2–0.5 ×

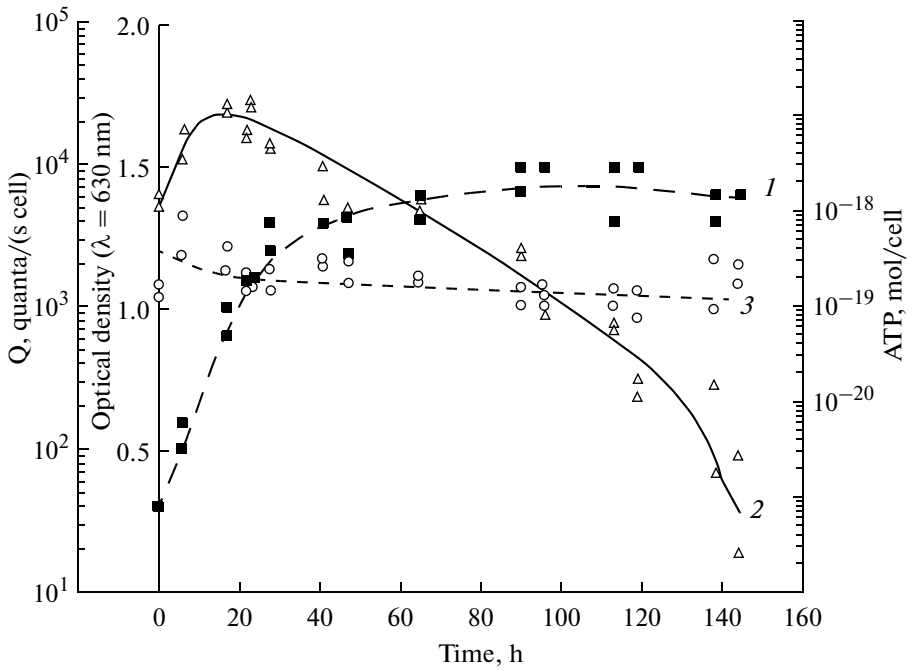


**Fig. 1.** The dynamics of growth (*1*), specific bioluminescence activity (*2*), and the ATP pool (*3*) in submerged culture of *P. phosphoreum* in the complex medium at 20°C.

$10^{-18}$ mole/cell) during the whole period of the luminescence cycle. Specific bioluminescence activity reached a maximum at 20 h, which was followed by a slow decay of luminescence. A decrease in activity during the 100 h period did not exceed 1.5 orders of magnitude.

Fig. 2 deals with the values in the tested parameters and the ATP pool in bacteria sampled at the stationary growth phase. Biomass with a cell concentration of $10^9$ cells/mL was incubated at 4°C in the cultivation medium without aeration and stirring. Under these incubation conditions and at the tested cell concentration, the respiratory chain was blocked due to oxygen deficiency. Accordingly, there was no reason why intense ATP synthesis should proceed in the dormant cells. The emission activity of bacteria under oxygen limitation was retained at a level of 10–15% of the maximum values. The results of analysis of the emission activity and of the ATP pool of the cell suspension revealed a complete lack of correlation between these parameters. The emission activity of the stationary-phase cells under the tested incubation conditions remained at the maximum level for 150 h, whereupon it monotonously attenuated; the decrease in luminescence during the 300 h period did not exceed 1.5 orders of magnitude. The intracellular ATP concentration did not change; it was 0.2–1.0 × $10^{-18}$ mol/cell.

Fig. 3 displays the luminescence dynamics and the ATP pool of the same cells upon incubation in a starvation medium (3% NaCl solution). In the absence of exogenous substrates, luminescence decay proceeded at a higher rate than in a nutrient-rich medium (Fig. 2). However, despite appreciable changes in emission activity, the ATP content of the cells did not change. During the 120-h incubation period, the ATP pool remained within the 0.4–0.6 × $10^{-18}$ mol/cell range.

Therefore, limitation of the emission process by oxygen or energy substrate deficiency (Figs. 2, 3) was

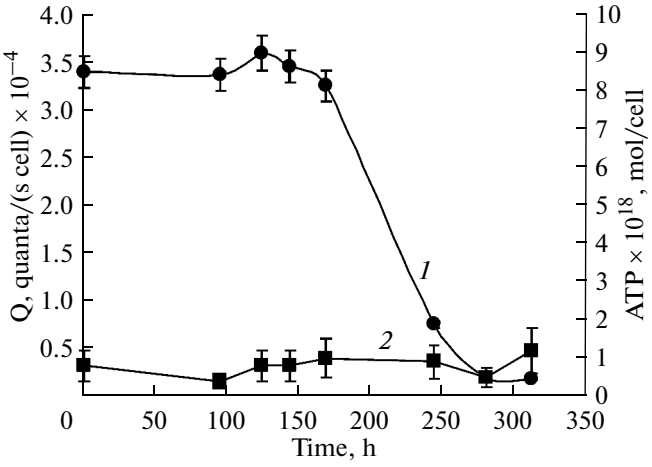


**Fig. 2.** The dynamics of specific bioluminescence activity (*1*) and the ATP pool (*2*) during the incubation of stationary-phase cells in the cultivation medium at ~4°C. Luminescence intensity and ATP concentration were normalized by dividing by cell concentration and expressed as absolute photon yields (quanta/s × cell) and specific ATP concentrations in the cell (mol/cell).

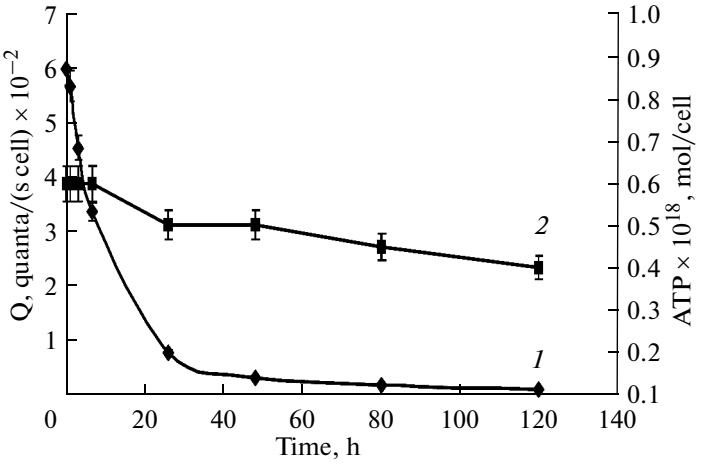


**Fig. 3.** The dynamics of specific bioluminescence activity (*1*) and the ATP pool (*2*) during incubation of the stationary-phase cells in 3% NaCl solution. The conditions were analogous to those given in the legend to Fig. 2.

not accompanied by a decrease in ATP concentration in the stationary-phase cells during the entire emission process.

The following experiments were conducted with PVA cryogel-immobilized bacteria. Application of this system was due to the following factors. Energy leakage resulting from alternative electron transfer pathways may be ruled out in the case of immobilized cells; in the energy balance equation, the metabolic energy is only distributed among the irreversible light and the reversible dark life-sustaining processes in the cell. The method of immobilizing in PVA cryogel we developed [9] results in no serious stress for photobacterial cells, with their viability up to 100%. It was therefore a sensitive system for assessing the metabolic activity and energy characteristics of the cells. If the granules were incubated at 4°C for two weeks, no medium luminescence changes were observed; this indicated that cells did not diffuse from the gel into the incubation medium. Immobilized photobacterial preparations were characterized by significantly increased luminescence stability compared to free cells, which made it possible to analyze their energy parameters for a prolonged period.

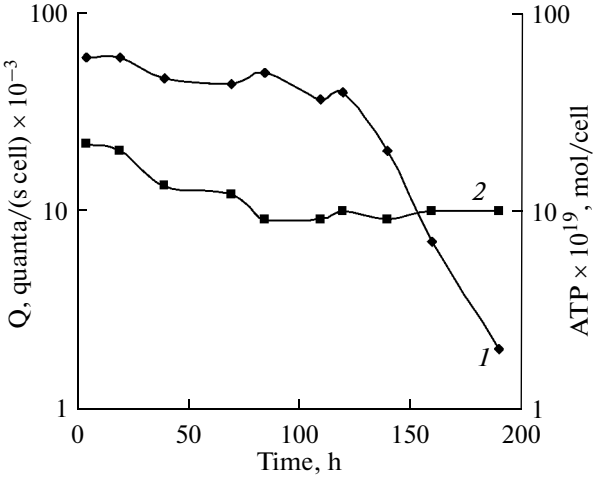


**Fig. 4.** The time course of bioluminescence activity (*1*) and the ATP pool (*2*) in PVA cryogel-immobilized *P. phosphoreum* cells (~$10^6$ cells/granule) incubated at 4°C in the cultivation medium.

Fig. 4 presents the results of our analysis of luminescence and ATP concentration in immobilized bacterial preparations (with a cell content of $2 \times 10^7$ cells per 1 granule) during incubation in the cultivation medium. At 4°C, a stable emission level was maintained for 100 h, and further incubation of the granules resulted in luminescence decay period; subsequently, stable emission with a low photon yield (~$10^{-4}$ of the original value) was observed. Under the tested conditions, luminescence was still detected after incubating the preparations for one month. The results of a parallel analysis of luminescence and ATP content in immobilized cells during a 200-h period indicated a lack of relationship between these parameters. While luminescence decreased by 3 orders of magnitude, the ATP content of the preparations remained virtually constant; it only varied within the limits set by the structural characteristics of the research subject, the extraction protocol, and the ATP assay technique.

It is known that the bacterium *P. phosphoreum* is capable of maintaining cell metabolism and growth at low temperatures (down to 4°C). The maintenance of a constant ATP pool, along with endogenous substrates and medium components, can be attained by immobilized cells which are incubated in the cultivation medium. If immobilized cells are incubated in a medium lacking energy and a carbon source, then the cellular energy metabolism is limited by the endogenous substrates. Comparative analysis of the bioluminescence activity and the ATP pool of immobilized

cells with granules (~$10^7$ cells per granule) incubated in 3% NaCl (1/20 v/v) is shown on Fig. 5.

These results indicated that the kinetic profile of emission by immobilized cells incubated in saline differed from that of the granules incubated in the cultivation medium (Fig. 4). Although the temporal parameters of the luminescence in various media exhibited close values, emission stability was significantly decreased in the saline medium: the maximum luminescence level was maintained for up to 2 days, whereupon emission activity decreased exponentially. The ATP content of immobilized cells remained virtually unchanged during the 10-day incubation period, whereas the luminescence activity of immobilized cells decreased by two orders of magnitude during the same period. The absolute luminescence and ATP content values were close to those obtained in a rich medium containing peptone, yeast extract, and glycerol (Fig. 4). This finding indicates that bacteria immobilized in cultivation medium-based carrier are sufficiently provided with the donors and cofactors of the bioluminescence process for the whole luminescence cycle.

Hence, immobilized preparations of photobacteria retained a constant ATP pool while incubated in both rich and starvation media. Like free cells, they revealed a complete lack of relationship between emission activity and the ATP content of the cells.

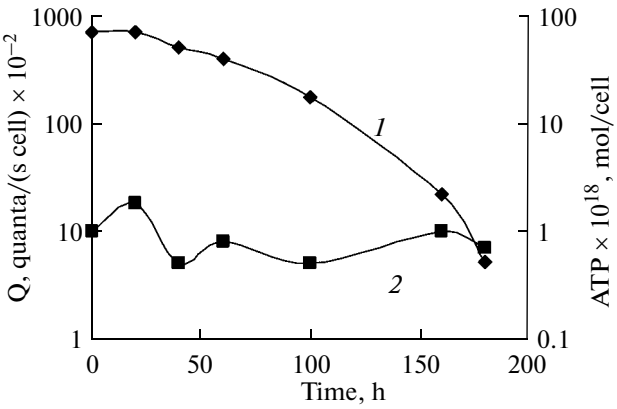


**Fig. 5.** The time course of bioluminescence activity (*1*) and the ATP pool (*2*) in PVA cryogel-immobilized *P. phosphoreum* cells (~$10^7$ cells/granule) incubated at 4°C in 3% NaCl solution.

## DISCUSSION

The factors that control the luminescence cycle of a growing culture of photobacteria have not been elucidated yet. The time profile of the luminescence cycle may have a luminescence system-specific pattern, which is primarily due to (i) energy storage via the formation of a reduced flavin in the NADH-dehydrogenase reaction [16] and of an aldehyde in the NADPH, ATP-dependent reductase reaction [17] and (ii) ATP consumption associated with the cell energy metabolism. In [18], the conclusion was drawn that transition to the stationary phase may be linked to the uncoupling of electron transfer in the luminescence electron transfer chain because of the synthesis of alternative electron acceptors. The luminescence decay may be significantly influenced by a downward pH shift, which decreases the rate of the FMN reductase reaction [7]. According to the work [19], luminescence decay may be due to luciferase inactivation by instable factors produced or accumulated during the stationary phase. Biosynthetic reactions also compete with the luminescence process. At least $2 \times 10^6$ ATP molecules/cell are required for protein synthesis in a typical bacterial cell [3].

The results of this work provide evidence that the ATP pool in the psychrophilic bacterium *P. phosphoreum* is maintained at a virtually constant level during the course of the whole luminescence cycle (over 100 h) (Fig. 1). The same phenomenon occurs during the incubation of stationary-phase cells both in the substrate-enriched medium and the starvation medium (3% NaCl) (Figs. 2, 3). The ATP content revealed is within the range reported in the literature [4, 8]; it amounts to $0.1–0.5 \times 10^{-18}$ mol/cell. Our results are consistent with the data of the work by Karl and Nealson [8] that also documented the existence of a constant ATP pool in *Vibrio harveyi* (MAV) and in the brightly luminescent strains of *V. fischeri, P. phosphoreum*, and *P. leiognathi*. The constant size of the ATP pool indicates that the level of dark leakage is low and energy consumption for life-sustaining metabolic processes is insignificant in nondividing bacteria in submerged culture and in the stationary-phase, proliferatively inactive photobacterial cells. Stability of the ATP pool during long-term storage suggests the absence of ATP leakage or uncoupling of oxidative phosphorylation as was reported in [18]. A constant size of the ATP pool was observed in PVA cryogel-immobilized cells in preparations incubated for over 150 h. The absolute ATP content values insignificantly varied in different incubation systems (Figs. 4, 5). The discrepancy in the luminescence decay kinetics between immobilized cells incubated in 3% NaCl and those incubated in a rich medium were not attributed to energy substrate deficiency in the bioluminescence system because the luminescence duration was approximately equal in both systems. We reported previously [13] that an acidic pH shift during the incubation of the preparations, which decreases the rate of $NAD^+$ reduction by $NAD^+$-dependent dehydrogenases, is the main factor causing luminescence decay in submerged cultures. In all likelihood, the differences in the bioluminescence decay dynamics of free and immobilized cells in various media are due to the differences in the rate of the pH shift, which is more rapid in saline than in a buffer-containing medium.

According to the results obtained, the statement can be made, therefore, that luminescence decay both

in the growing culture and in the stationary-phase and immobilized cells is not due to a decrease in the cellular membrane potential [20]. The long-term stability of the ATP pool confirms the validity of bioluminescence-based ATP measurements for quantitative assessment of both free and immobilized photobacteria, irrespective of their growth stage, medium composition, and the preparation's incubation time.

The results obtained are of relevance to the issue of the formation of the aldehyde co-substrate of the luciferase and its involvement in the bioluminescence process. It is generally accepted [2, 3] that the bacterial luciferase functions as a monooxygenase: it catalyzes the cooxidation of reduced flavin and a long-chain ($C_8$–$C_{16}$) aliphatic aldehyde by molecular oxygen

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + H_2O + h\nu. \quad (1)$$

During the reaction, the aliphatic aldehyde is oxidized to the respective acid.

It is assumed that the main pathway of the formation of aliphatic aldehydes in the cell is the process in which RCHO results from the NADPH, ATP-dependent reduction of a fatty acid by a fatty acid reductase [2, 21]:

$$RCOOH + ATP + NADPH \longrightarrow RCHO + AMP + PPi + NADP^+. \quad (2)$$

Nevertheless, the content of $C_{12}$–$C_{16}$ aliphatic aldehydes in the cell was estimated to be quite low in [22]; they can maximally sustain the light reaction for 1 s. If the emission is intense ($10^4$–$10^5$ quanta $\times$ $s^{-1}$ $cell^{-1}$), as is the case with a number of strains of photobacteria, the aliphatic aldehyde (reaction 1) and, accordingly, ATP (reaction 2) should be utilized virtually instantaneously. Obviously, a potent ATP generation system with at least 100 turnovers per second is required in order to impose no limits on the luminescence. According to the data presented in the literature and our own findings, the ATP content is 0.2–2.0 $\times$ $10^{-18}$ mol per cell. The respiration rate in *P. phosphoreum* is 10–80 $\times$ $10^{-18}$ mol per cell [4, 8]; with P/O = 2, this is insufficient for attaining the ATP concentration required for the NADPH, ATP-dependent reductase reaction in which the aldehyde substrate is formed.

The data obtained in this work indicated that the integral photon yield in the growing cells of the psychrophilic bacterium *P. phosphoreum* amounts to $10^8$ photons per cell during a 100 h period; consumption of ~$10^{-16}$ mol per cell of luciferase substrates is required (assuming 100% efficiency). Energy substrate and oxygen limitation does not result in decreasing the ATP pool in the stationary-phase cells, which contrary to the expected result based on the utilization of the aldehyde substrate in the emission process. Integral analysis of the emission activity of immobilized cells revealed that the total number of photons emitted by the highly active preparations during a 200-h period amount to $10^9$ photons per cell and minimally $10^{-15}$ mol/cell of the substrates are consumed. Nevertheless, the ATP pool was maintained at a constant level ($10^{-18}$–$10^{-19}$ mol/cell) both in free and immobilized bacteria. The kinetic and quantitative data contradict the possible formation of the aldehyde substrate of the luciferase by the reduction of the respective fatty acid via the NADPH, ATP-dependent reductase reaction. Stability of the ATP pool indicates that the aldehyde is not utilized in the light reaction; therefore, the bacterial luciferase cannot be considered a monooxygenase. The difference between the bacterial luciferases and monooxygenases was emphasized in [23]. Based on the protein sequencing data and the kinetic characteristics, the authors drew the conclusion on substantial differences between bacterial luciferases and all known monooxygenases in phylogenetic and biochemical terms. Similar views are held by the authors of the work [24] in which they suggest that the role of the aldehyde in the bioluminescence reaction boils down to forming a part of the emitting complex.

Of special interest is the fact mentioned in [25] that the chromophore (FMN) is present as a covalently bound FMN-myristate derivative in the luciferase-associated flavoprotein ($FP_{390}$) emitter in *P. phosphoreum*. Based on these data, it may be suggested that the function of the long-chain aliphatic aldehyde in the bioluminescence process involves the formation of a flavin–alkenyl adduct that provides for efficient binding of the hydrophilic flavin substrate ($FNNH_2$) to the hydrophobic pocket of the active center of luciferase. In this case, only an insignificant amount of aldehyde is required; the aldehyde need not be removed from the enzyme complex. The operation of this mechanism does not rule out the possibility of aldehyde oxidation to the acid, on the assumption that the oxidation is a concomitant process.

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