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EXPERIMENTAL ARTICLES

Physiological and Emission Characteristics of the Luminescent Bacterium *Photobacterium Phosphoreum* from the White Sea

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Abstract—Growth and emission characteristics of the luminescent bacterium *Photobacterium phosphoreum* strain KM MGU 331 originating from the White Sea and isolated from the intestine of a bottom-dwelling fish, the European sculpin, *Myoxocephalus scorpius*, were analyzed. The strain is characterized by a high rate of colony formation and high intensity of light emission on agarized medium at 4°C as well as by highly efficient (5×10^5 quanta s^{-1} $cell^{-1}$) and prolonged (over 100 h) light generation upon submerged cultivation at 20°C. The acidic shift of pH in the medium didn't exceed 0.3 pH units. Effects of temperature, pH, and sodium chloride concentration on emission characteristics of intact photobacterium cells were studied. The optimal temperature for luminescence was found to be 15°C. The maximum luminescence activity was stable in a wide pH range from 7.0 to 9.0. Luminescence occurred within the range of 0.2–3.8% NaCl with the maximum at 2.5%. The results obtained confirm the literature data suggesting that luminescent bacteria adapted to low-temperature conditions possess a highly conjugated system of electron transfer to luciferase.

Key words: *Photobacterium phosphoreum*, luminescence, cultivation, temperature, pH, sodium chloride.

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Ecological distribution of marine photobacteria correlates strictly with the temperature profile of sea water [1–3]. Direct correlation was demonstrated between ocean depth, temperature gradient, and the taxonomic composition of photobacterial species [4–6]. Below 200 m and 15°C, psychrophilic *Photobacterium phosphoreum* strains were dominating while in the upper water layers the mesophilic species *Vibrio harveyi* was preponderant.

Besides other morphologic and physiologic characteristics, psychrophilic photobacteria *P. phosphoreum* are distinguished by the most intense and most lasting luminescence [1, 7]. Specific activity of described *P. phosphoreum* strains is the highest among other species, up to 5×10^4–10^5 quanta s^{-1} $cell^{-1}$, which is an order of magnitude higher than the values for the mesophilic strains of *V. harveyi* and *V. fischeri* [8, 9].

Data available on photobacteria cultivation allows distinguishing of the predominating physiological processes in the multiple controls of growth and luminescence. The most important among these factors are the reducing potential of the cell, ATP pool, and coupling of the electron transfer chain with luciferase, as they support the reduced state of the flavin substrate and provide the biosynthesis of the aldehyde substrate. These processes are largely influenced by a pH shift produced by acidic metabolites of growing bacteria. Autocatalytic acidification of the medium by a growing population suppresses emission activity [1, 8]. Depending on the species and strains, the value of the shift varies from 0.5 to 2.0 pH units [9, 10]. It is important that the rate of acidification is significantly higher in the dark mutants than in the wild strains [10].

Temperature, pH, and sodium ions exhibit a direct effect on the activity of bacterial emission. However, the optimum values for pH, salinity, and temperature vary between species and even strains of photobacteria [5–7]. The temperature range of 2–30°C with the maximum at 15–18°C is characteristic of light emission by most *P. phosphoreum* strains [7, 9]. At temperatures exceeding the optimum values by 10–15°C, partial or complete loss of bioluminescence occurs [12]. The nature of the phenomenon is not studied yet. Most of the works are either limited to the description of the luminescence characteristics as a function of temperature [2, 6, 11] without going into details of the effect of temperature on cell structures or reduces the problem to the declaration of dark mutant formation [12]. Moreover, the issue of the critical values of "temperature/time" doses for reversible and irreversible effects on light emission in various photobacteria species and strains remains unconsidered.

Optimum pH values for different *P. phosphoreum* strains vary between 7.0 and 8.5 [7, 11, 13]. Along with strain characteristics, these differences may be due to the buffer composition and physicochemical conditions of the measurement procedure. Generally, the dependency between the salt concentration and the lumines-

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cence is observed between 0.5 and 6% NaCl [7, 11, 13] and varies between different *P. phosphoreum* strains.

Altogether, these factors shape the luminescence cycle, its duration, and intensity being specific for a given bacterium species. The luminescence cycle in *V. harveyi* is short (12–14 h) and it finishes completely during the logarithmic growth phase; in *V. fischeri* it lasts for 20–24 h, while in *P. phosphoreum* stable luminescence is the most continuous (1.5–2 days) [10, 13]. Physicochemical characteristics of bacteria of different species reflect their adaptation to the physicochemical conditions of their environment [3, 4, 6].

On the basis of temperature control and data of emission parameters for different photobacteria species, one may expect existence of strains with high-efficient and prolonged luminescence not only in ocean depth but also in the surface area of the water basins with a low average annual temperature. The temperature conditions of the White Sea are optimal for psychrophilic bacteria. The temperature of its surface waters does not exceed 15°C in summer.

In the present work growth and luminescence characteristics of a luminescent bacterium isolated from the intestine of theWhite Sea fish are described.

MATERIALS AND METHODS

The luminescent bacterium *Photobacterium phosphoreum* strain KM MGU no. 331 was the subject of the study. It was isolated from the intestine of a European sculpin, *Myoxocephalus scorpius*, inhabiting the tideland of the Kandalaksha bay of the White Sea. For comparative analysis of emission characteristics, *P. phosphoreum* strain ATCC 11040 was used.

Bacteria cultivation. Bacteria were cultured on an agarized medium (1.2%) containing the following (g/l): nutrient agar (Obolensk, Russia), 12.5; NaCl, 28.7; $MgCl_2 \cdot 7H_2O$, 4.5; $CaCl_2$, 0.5; KCl, 0.5; yeast extract, 1.0; pH 7.6, at 4°C. For a submerged culture in liquid medium, the medium contained 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 7.6, at 20°C on a rotary shaker (200 rpm, flasks of 700 ml containing 150 ml of culture medium). The inoculum was grown on an agarized medium for 24 h at 4°C, washed off with 2% NaCl, and introduced into the culture medium as 5-ml aliquots (5×10^9 cells ml^{-1}) per flask.

Growth parameters were controlled by optical density at 660 nm on a Beckman-26 spectrophotometer (United States). Cell concentration was determined using the optical density–cell number calibration curve. Luminescence was registered on an LKB-Wallac 1250 lumenmeter (Sweden–Finland). Luminescence intensity–temperature curves were recorded on an RTF-20046 impulse counter (Germany) equipped with a thermostatic cuvette compartment. Temperature was measured with thermometer probes introduced directly into the bacterial samples. Emission intensity was expressed in relative units. For quantitative analysis, the lumenmeter was calibrated using the Hastings–Weber standard [14]. A standard pH electrode on a pH-340 pH-meter was used for pH measurements. Bacterial cells were precipitated by centrifugation (4000 *g*, 20 min) and single washing with 0.1 M sodium phosphate buffer, pH 7.6 supplemented with 2% NaCl. Washed cells were resuspended in the buffer medium of the same composition and stored at 4°C. To determine pH dependency of cell luminescence, 0.1 M sodium phosphate–carbonate buffers with various pH values were used, supplemented with 2% NaCl. The effect the salt concentration was determined in the range of 0.2–6% NaCl. Emission activity in this case was measured at room temperature after emission achieved the maximum level.

All experiments on growing culture and intact cells were performed in three repeats. Statistical treatment of the data was performed using the standard Statistica for Windows 5.0 software package; the Student's *t*-criterion was applied considering the differences significant at $p \leq 0.05$.

RESULTS

The White Sea's photobacterium strain grown on agarized medium at 4°C demonstrated highly efficient growth with intense emission 14–16 h after inoculation, which is at least 3 times higher than in the case of ATCC 11040. Emission intensity of the colonies was determined in the biomass washed off with cool (4°C) culture medium recalculated per cell according to the optical density–cell number calibration curve. The luminescence activity of bacteria at 4°C was 10% of the activity at room temperature. The colonies luminescence at 4°C was observed for as long as 1 month.

Growth and emission curves obtained upon submerged cultivation at 20°C are presented on Fig. 1. The logarithmic growth phase lasted a little more than 1 day under these conditions and the stationary phase, up to 60 h.

The level of light emission increased proportionally to the increasing number of bacteria and was retained for 3 days in the stationary culture.

The decrease of emission activity in post-stationary cultures was less than two orders of magnitude compared to the maximum.

Bioluminescence activity of bacteria under optimized conditions of submerged culture (22 h growth at 20°C) reached ~5×10^5 quanta s^{-1} cell^{-1},which exceeds the values reported for other strains of the species [7–9]. The data were additionally confirmed by comparative analysis with the known strain ATCC 11040 whose luminescence activity under the same conditions was 5×10^4 quanta s^{-1} cell^{-1} (data on submerged cultivation of the strain are not presented).

The specific light-emission activity practically didn't change during the exponential growth phase,

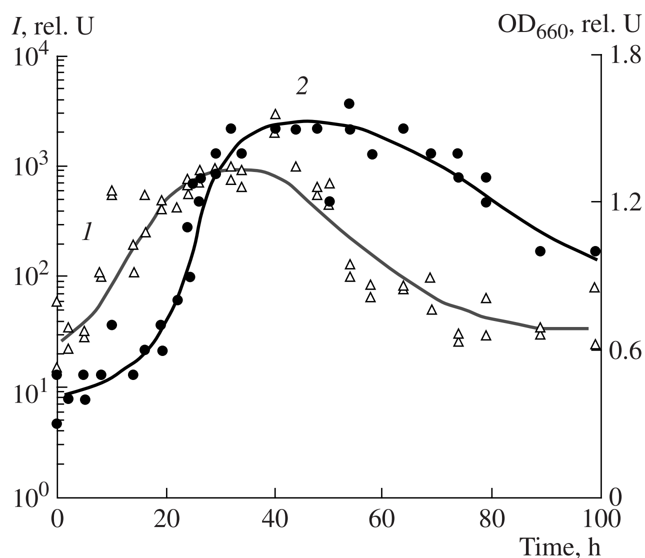

Fig. 1. Dynamics of luminescence (*1*) and growth (*2*) of bacteria in submerged culture at 20°C. The composition of the medium is described in Materials and Methods.

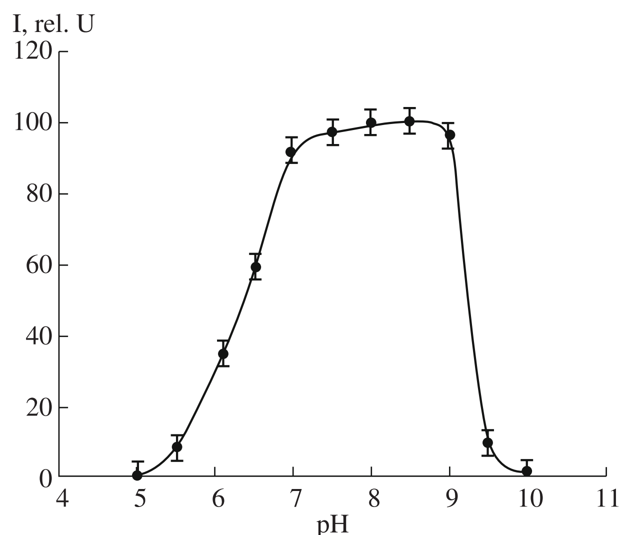

Fig. 3. Luminescence of intact photobacterium cells as a function of pH in 0.1M phosphate–carbonate buffer with 2% NaCl at 22°C.

suggesting the absence of autoinduction in the process of de novo luciferase synthesis. This is typical of the species *P. phosphoreum* [8, 9].

Under submerged cultivation in the described medium with an initial pH value of 7.5, the pH shift towards acidification over the whole period of growth was insignificant, from pH 7.5 to pH 7.2; the pH changes occurred during the exponential growth phase (Fig. 2).

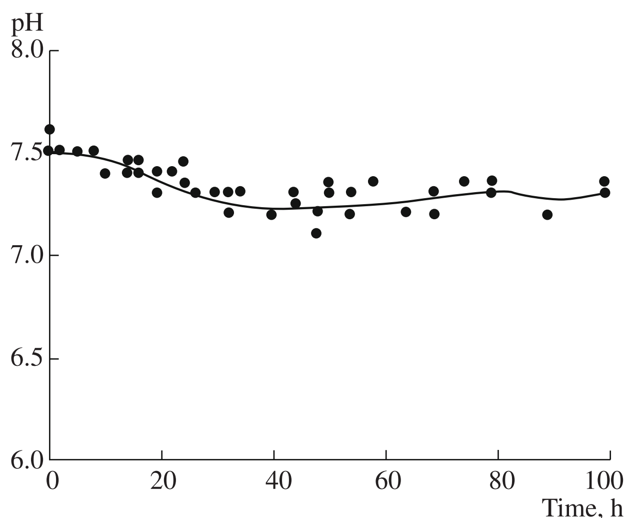

Fig. 2. Kinetics of pH shift of the growth medium in submerged culture at 20°C.The composition of the medium is indicated in Materials and Methods.

Data on light emission by the exponential-phase intact cells depending on pH, salinity, and temperature are presented below.

The luminescence of intact cells in 0.1M phosphate–carbonate buffer depending on pH is presented in Fig. 3. Pronounced tolerance to alkaline conditions was established. The maximum of the luminescence was observed within a wide pH range, from pH 7.0 to pH 9.0, with the luminescence activity decreasing at pH values below 6.5 and above 9.0.

The profile of cell luminescence at different NaCl concentrations is presented in Fig. 4. The maximum light-emission activity was registered in a very narrow range at 2.5% NaCl concentration, and the luminescence was relatively high in 0.5% NaCl. A sharp decrease was observed at NaCl concentrations above 4%.

The luminescence–temperature profile of intact cells was typical of psychrophilic bacteria with the luminescence maximum at 15°C (Fig. 5). A high emission level (20%) was retained at low temperatures down to 2°C.

In the process of direct scanning from 2 to 35°C at a rate of 1.8°C min^{-1}, the residual activity at 35°C was 0.01% of the luminescence activity at 15°C, while the same value upon heating up to 25°C was 50%.

Loss of luminescence upon heating above 30°C is irreversible, which was confirmed by reverse scan towards lower temperatures. After heating up to 35°C at 1.8°C min^{-1}, cooling at the same rate restored only 0.01% of the activity of cell luminescence.

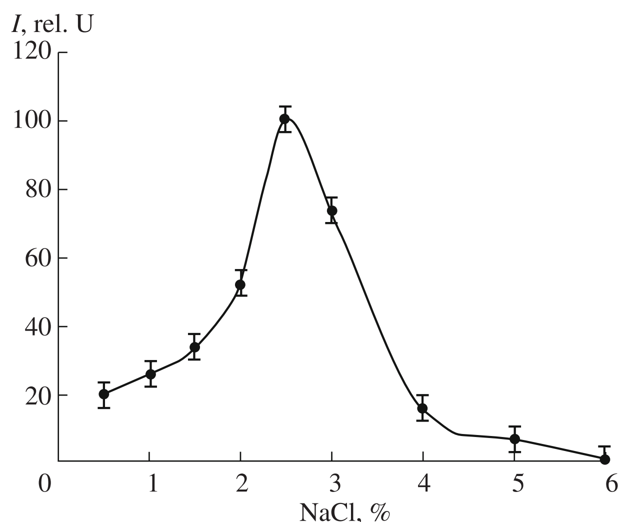

Fig. 4. Emission activity of intact photobacterium cells in NaCl solutions of varying concentration.

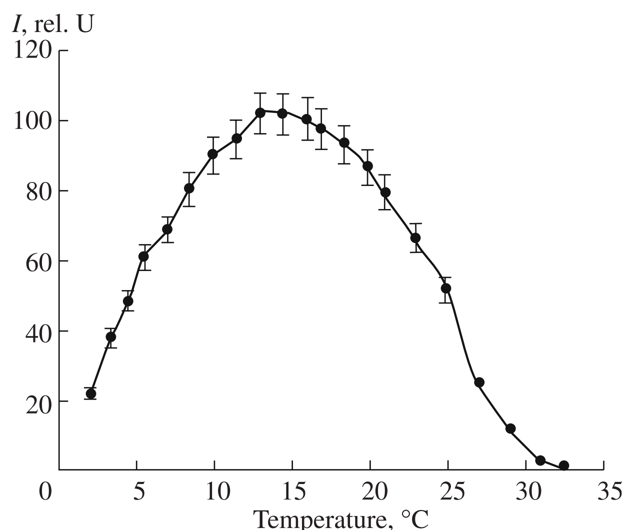

Fig. 5. Luminescence of intact photobacterium cells as a function of temperature in 0.1M phosphate–carbonate buffer with 2% NaCl, pH 7.5.

DISCUSSION

A psychrophilic luminescent bacterial strain of *P. phosphoreum* isolated from the intestines of a bottom-dwelling fish, a European sculpin, *Myoxocephalus scorpius*, inhabitant of the Kandalaksha Bay of the White Sea, exhibited highly intense and stable luminescence of values exceeding those reported so far for the brightly luminescent *P. phosphoreum* strains isolated mainly from ocean depth [2–5]. The results obtained indicate the dominating role of temperature and NaCl concentration in the energetic and physiological adaptation of luminescent bacteria and are also in good accordance with the data [4–6] on the depth distribution of photobacteria species.

Low-temperature adaptation is expressed primarily in high intensity of emission and a relatively high growth rate at 4°C. At the same time, the temperature maximum of the luminescence (15°C) corresponds to the values reported in the literature for many other strains of this species from other sea regions [4, 11, 13].

The duration of the luminescence cycle at submerged cultivation of the studied psychrophilic strain at 20°C was over 100 h, and the decrease of the luminescence activity during the period didn't exceed 2 orders of magnitude compared to the maximum values.

Similar results were presented in the work [16]. The authors of this work performed statistical optimization of the composition and cultivation conditions of *P. phosphoreum* strain KCTC 2852 with the result that the bioluminescence duration in a submerged culture until its complete fading increased up to 80 h.

The emission characteristics of the cells under submerged cultivation conditions support the hypothesis suggested in [15] that psychrophilic photobacteria posses a more conjugated system of electron transfer to luciferase than the mesophilic ones, which is manifested through a longer and more intense luminescence cycle of the submerged culture.

A requirement for highly efficient conjugation of the electron transfer system with the luciferase reaction is the most important factor determining emission activity as it is in connection with the need in maintenance of high reduction rates of the luciferase flavin substrate ($FMNH_2$ pool) in the NADH-dehydrogenase reaction to prevent nonenzymatic oxidation by the oxygen of air [9, 17]. Decrease in the rate of flavin reduction in a growing culture may be due to a general decrease in the rates of NAD-dependent dehydrogenase reactions owing to a pH shift towards acidic values and (or) to disjunction of the system of electron transfer to luciferase in the process of cultivation [17, 18].

In the work of Watanabe et al. [10], a direct relationship was established between the duration of the luminescence cycle and the rate of acidification of the medium in the case of microbial growth.

A low rate of acidification of the medium in the process of submerged cultivation and wide pH range for the maximum luminescence activity are characteristic of the strain under study. Together, these two factors provide stable luminescence of a submerged culture. The initial pH values may vary between pH 7.0 and 8.5. The pH dependency of the luminescence data is in accordance with the data presented in [19], which reports that *P. phosphoreum* strain MT 10201 isolated from the luminous organs of a *Phisiculus japonicus* fish is characterized by active growth at pH between 6.0 and 9.0 and the luminescence maximum is at pH 8.5.

Analysis of the luminescence profile as a function of temperature demonstrates that heating bacteria for less than an hour at 15°C above the temperature optimal for

luminescence induces an irreversible loss of cell luminescence. The most probable reason for this phenomenon may be the temperature-dependent disjunction of the electron transfer chain and luciferase [18].

The luminescence profile as a function of salinity demonstrates that the luminescent activity of the cells is retained at a high level in a narrow range of NaCl concentrations, between 2 and 3%. A sharp decrease in activity was observed at the NaCl concentration of 4% and higher, suggesting the adaptation of this strain to the low salinity of the White Sea (24–26‰). The luminescence of many *P. phosphoreum* strains originating from the environments with higher salinity remained stable in the presence of up to 5–6% NaCl. In particular, this is characteristic of *P. phosphoreum* strains 11040 from the North Sea [13] and 10201 from the Sea of Japan [19].

Altogether the data obtained is evidence of an adaptation of energy metabolism of the luminescent bacterium to the temperature and salinity conditions of the White Sea. The results support the literature data [10, 15] stating that psychrophilic luminescent bacteria *P. phosphoreum* possess a highly conjugated luciferase electron transfer system.

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