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Influence of ultrahigh frequency irradiation on Photobacterium phosphoreum luxb gene expression

Research Article

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Abstract: Continuous increase in the number and the variety of anthropogenic sources of electromagnetic radiation causes a high interest in studying the effects ultrahigh frequency on living organisms. In the present research influence of UHF EMR (15 W, 2.45 GHz) for 5 and 15 min on morphological and genetic peculiarities of Photobacterium phosphoreum colonies was studied. It has been revealed that UHF EMR affected colony growth parameters, induced transcriptional activity of luciferase encoding gene expression and that the effect was depended on exposure duration. The subsequent cultivation of bacteria during a two week period after treatment showed maintaining of the increased luxb mRNA level in irradiated colonies. Opposite bacterial stress responses were detected to UHF EMR and elevated temperature treatments that assumed UHF EMR comprised of not only thermal but specific component of non-thermal nature.

Keywords: *Ultrahigh frequency electromagnetic radiation • Photobacterium phosphoreum • Bioluminescence • Luxb gene level*

Abbreviations:

Ct – threshold cycle;
qRT-PCR – quantitative reverse transcription PCR;
UHF EMR – ultrahigh frequency electromagnetic radiation.

1. Introduction

Natural and anthropogenic non-ionizing radiation pressure on the biosphere has strengthened recently due to the significant environmental deterioration and a sharp increase in the use of various electrical devices [1]. The problem of "electromagnetic compatibility" of nature and sources of electromagnetic radiation (EMR) is becoming increasingly important [2]. To date, significant correlation between the activity of this factor and the vital functions of various living beings are being established [3]. Microorganisms are widespread in nature and form the basic ecological and trophic links in the functioning of the biosphere. According to the results of the studies conducted over the past 20 years, we should expect significant changes in natural microbial associations as a result of man-made electromagnetic radiation generated by diverse electrical equipment. Multidimensional implementation of advanced electromagnetic devices into the daily lives violates the natural electromagnetic background, which according to the modern concepts is an integral part of the development and existence of living matter [4].

Thus, the study of biological effects of non-ionizing electromagnetic radiation on microorganisms is relevant and has both theoretical importance in identification of the mechanism of EMR on microorganisms and practical – in using the acquired knowledge in biotechnology to protect the environment and human health.

Assay systems based on luminous bacteria are good candidates for monitoring environmental toxicity. The tested parameter here is bacterial

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luminescence intensity, which can be easily measured [5]. Bioluminescence intensity of luminous bacteria reflects various factors to solve a variety of problems [6]. Numerous applications of the bacterial bioassay are known [7-13]. The advantages of the bioluminescent assays are speed, sensitivity, simplicity, and availability of the devices for toxicity registration [14].

Bacterial luminescence intensity directly depends on luciferase activity. The enzyme consists of two subunits, α and β, and the later provides the high quantum yield of the reaction [15]. It is known that luminescence depends on cell density value [16] which, in turn, relates to the increase in luxb mRNA level. In previous studies a correlation between *P. phosphoreum* cell density and light emission has been shown [17]. One of the perspective directions of expansion of bioluminescent analysis is its use to assess the level of electromagnetic radiation [18]. Studies of luminescence dynamics in photobacteria liquid culture subjected to EMR revealed changes in luminescence activity with duration of exposure and the age of bacterial culture [19].

In the present study we evaluate the impact of UHF EMR on morphological and genetic characteristics of *P. phosphoreum* and its effect on the growth of bacterial colonies. We also compare the influence of UHF EMR and elevated temperature, as the constituent of UHF EMR, on *P. phosphoreum* growth and luciferase gene activity.

2. Experimental Procedures

2.1 Bacteria, culture conditions

The luminous bacterium *Photobacterium phosphoreum* used in our research were isolated from *Squalus acanthias* inhabiting the Black sea and placed in the depositary of microorganisms of Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine as IMV-7071.

The identification of bacteria was performed using morphological, cytological and biochemical tests in previous studies [20]. To confirm previous identification 16S rRNA gene sequencing was performed. Total DNA from cell suspension was isolated using Proba-CTAB (DNA-technology, Russia) DNA isolation kit according to manufacturer' instruction. 16S rRNA gene was amplified with primers 27F and 1492r, sequencing procedures and data analysis was performed as described previously [21].

Bacteria were grown for 8 hours in liquid media composed of (g L^{-1}): peptone - 5.0, yeast extract - 1.0, NaCl - 30.0, Na2HPO4 – 5.3, KH2PO4 x 2H2O – 2.1, (NH4)2HPO4 – 0.5, MgSO4 x H2O – 0.1, glycerol – 3.0 ml L^{-1} [22] at 22°C. Liquid bacterial culture was diluted to a concentration of 2 x 10^7 cells/ml and drops of equal volume (10 µl) were placed on solid agar medium in a Petri dish. Each plate was inoculated with 10 bacterial colonies and all experiments were performed in triplicates.

2.2 Treatment by ultrahigh frequency radiation

Microwave therapy apparatus "Ray-11" (Medical Equipment Factory, Russian Federation) was used as a source of electromagnetic radiation. 24 hour-old bacterial cells were subjected to a single exposure at a wavelength of 12.5 cm (corresponding to a frequency of 2.45 GHz) and power of 15 W for 5 and 15 min. Since electromagnetic radiation of ultrahigh frequency is accompanied by heating of the object, we also performed incubation of bacterial cells at 42°C instead of irradiation. All bacteria were cultivated at normal conditions on solid medium for two weeks after single impact.

2.3 Measuring of bacterial growth dynamics

The images of Petri dishes with bacterial colonies were captured with an Olympus digital camera SP560UZ under day light and in the dark. Colony size measurements were performed on the photographs either manually or using the program ImageJ 1.41 (http://rsb.info.nih.gov/ij/).

Bacterial colony diameter was measured in two directions on the 1st, 7th and 14th day. The colony area (S) and the radial growth rate (Kr) were calculated using formulas derived in [23]:

$$S = \pi \times D1 \times D2 / 4,$$

where π = 3.14, D1 and D2 – bacterial colony diameters (measured in two perpendicular directions) and

$$Kr = 0.5 \times (D1 - D0) / (t1 - t0),$$

where D0 and D1 are the bacterial colony diameters at time t0 and t1.

The area of colony outer cell zone was calculated by subtracting the area of central zone from colony general area.

The statistical significance between samples was calculated using Student's t-test and a $p < 0.05$ level of statistical significance was applied.

2.4 RNA extraction, cDNA synthesis and qRT-PCR analysis

Samples for genetic analysis were taken at three time points: immediately after exposure (1), 7 days (2) and 14 days (3) after treatment. Total RNA was isolated from bacterial colonies using RNA isolation kit Ribo-sorb (AmpliSens, Russia) according to

manufacturer' instructions. Residual DNA was removed with DNase I treatment as per the supplier's protocol (Fermentas). RNA concentrations were measured using "BioPhotometer" (Eppendorf, Germany). 1 μg of RNA was transcribed into cDNA using a cDNA synthesis kit (AmpliSens, Russia), containing a mix of random primers and M-MLV reverse transcriptase.

The reaction mix for qRT-PCR (total volume of 25 μl) contained 12.5 μl 2x Maxima SYBR Green/ Fluorescein qPCR Master Mix (Fermentas), 20 pmol of each primer and 3 μl of cDNA template. Each run had no template control and three dilutions of cDNA for the standard curve to calculate the coefficient of determination (R^2). Results of amplification were analyzed if R^2 was greater than 0.95, in other cases the reaction was repeated. Synthesis of cDNA and qRT- PCR were performed in duplicate for each gene and sample. qRT-PCR was performed in a qTower 2.2 thermal cycler (Analytik Jena AG, Germany) under following conditions: 95°C for 10 min; 40 cycles of 95°C for 5 s, 57°C for 5 s and 72°C for 10 s. 16S rRNA gene was considered as endogenous reference. Relative gene expression level was calculated with $2^{-\Delta\Delta Ct}$ method [24] and tested for statistical significance with t-test.

Primer sequences for the 16S rRNA (5'-ctgtcgtcagctcgtgttgt-3' and 5'-ttcatggagtcgagttgcag-3') and luxb (5'-agcatcagttcccactgctt-3' and 5'-cagtaccaatcgcatgttgg-3') genes were designed from the genome sequences published for *P. phosphoreum* with the online program Primer3. Primer specificity and dimer formation were checked by agarose gel electrophoresis and melting curve analysis.

3. Results and Discussion

To confirm previous identification made for bacteria used in our research we performed 16S rRNA gene sequencing. Totally, fragment of 1361 bp was sequenced and submitted to the GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov/genbank) under accession number KF656787. Results of BLAST analysis revealed 100% similarity with *P. phosphoreum* 16S rRNA gene fragments deposited in the GenBank database.

3.1 Colony growth parameters under UHF irradiation

Analysis of bacterial colony growth parameters showed that the radial growth rate of bacteria was higher in all tested samples compared to the control during the cultivation period and the biggest value was detected in colonies irradiated to UHF EMR for 5 min (15 W, 2.45 GHz, 5 min) (Table 1). The values of colony growth rate detected in colonies subjected to incubation at 42°C and irradiated to UHF EMR for 15 min were similar. The colony radial growth rate declined by 14th day in both control and treated bacteria.

The study showed differences between values of colony area calculated in control and treated samples (Table 2). The colony area of irradiated bacteria was larger than non-irradiated bacteria. Morphological analysis revealed the following changes: compared to control values on the 7th day of cultivation after UHF EMR with power of 15 W at 5 min the area of colonies was larger by 33%, and 15 min exposure resulted in area enlargement by 16%. After 14 days of cultivation,

Sample	1st day	7th day	14 th day
Control	2,58±0,19	14,64±1,13	21,80±1,94
42°C	1,20±0,14*	12,02±0,61*	21,42±1,20
UHF EMR, 5min	2,96±0,38	19,43±2,22*	31,83±2,44*
UHF EMR, 15min	2,86±0,29	17,01±2,22	26,78±2,43

Table 1. The colony radial growth rate of *P. phosphoreum* after exposure to UHF EMR and incubation at elevated temperature (μm/h). Data are presented as a mean value ± standard error (n = 30). * – $p < 0.05$.

Sample	Time period, days	
	1st – 7th	7th – 14th
Control	7.411±0.477	2.813±0.449
42°C	8.080±0.351	3.810±0.216*
UHF EMR, 5min	9.033±0.881	4.271±0.304*
UHF EMR, 15min	8.006±1.026	3.616±0.253*

Table 2. The general area of *P. phosphoreum* colony after treatment with different factors (mm2). Data are presented as a mean value ± standard error (n = 30). * – $p < 0.05$.

the area of irradiated colonies rose by 46% and 22.8% at 5 and 15 min of exposure, respectively. Pre heating bacteria at 42oC led to decrease in colony area by 17.8% (7th day) and 1.7% (14th day) in comparison with control samples. Besides, by the 14th day of cultivation cell differentiation inside colonies was observed (Figure 1). There were two cell zones: the marginal cells that emitted light and the central "dark" cells. The area of the outer zone emitting light was larger than central region in control samples (57%), similar area of both zones was observed in irradiated colonies (50% – 53%) and in pre-heated bacteria luminous cell area was smaller than non-luminous zone (41.4%) (Figure 1, c – e).

Thus, summarizing results of colony growth analysis it seemed that the changes of colony growth peculiarities caused by UHF EMR comprised of thermal and non-thermal components depended on exposure duration – the longer the treatment the more essential participation of thermal factor.

3.2 Evaluation of luxb gene expression

In our study oligonucleotide primers to 16S rRNA and luxb genes were designed to determine gene expression level with qRT-PCR. The specificity of primers was verified with agarose gel electrophoresis and melting curve analysis. Single band was visualized on the agarose gel when amplification was carried out. The melting temperature of PCR-products was varied from 81.4 to 83.5°C for 16S rRNA and from 75.5 to 77.1°C for luxb genes.

Changes of gene expression in bacterial cells subjected to stress factors were revealed using qRT-PCR analysis. Quantitation of luxb mRNA level in *P. phosphoreum* cells immediately after exposure to power of 15 W with a frequency of 2.45 GHz at 5 and 15 min showed 8- and 12-fold enlargement of this index, respectively, in comparison with non-treated bacterial cells. On the contrary, luxb expression in heated colonies showed two times decrease.

Analysis of transcriptional activity showed that the level of luxb expression determined in control / unexposed/ *P. phosporeum* colonies during the cultivation period increased on the 7th day and declined after 14 days of cultivation (Figure 2). An increase of the gene expression on the 7th day was observed in bacterial colonies exposed to UHF EMR for 5 min and a decrease of this index in colonies irradiated at UHF EMR for 15 min. Results of transcriptional analysis on 14th day after treatment showed lowering the luxb mRNA level in exposed colonies although their values were higher than control one. There was quite different gene expression mode in colonies subjected to incubation at 42°C: the luxb activity decreased on the 7th day slightly and went up on the 14th day.

Thus, results of quantitative transcriptional analysis showed that irradiation of bacterial colonies to UHF irradiation affected expression of luxb gene and the level of its activity depended on the duration of electromagnetic exposure. Changes in gene expression observed after incubation of *P. phosphoreum* at 42°C instead of EMR treatment were less dramatic than those after UHF EMR.

There were many studies describing the influence of various physical and chemical stress factors on

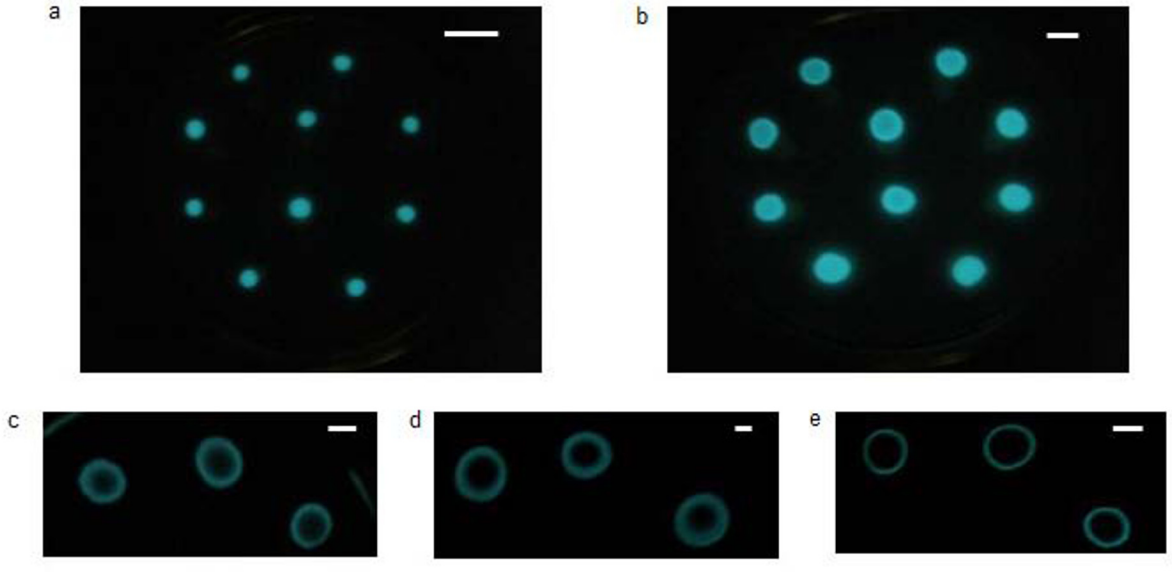

Figure 1. Images of *Pphosphoreum* colonies of different ages: control – 1 day (a); 7 days (b); 14 days (c); irradiated bacteria – 14 days (d); pre-heated bacteria – 14 days (e). Images were captured with an Olympus digital camera SP560UZ in the dark. The bar is equal to 6 mm.

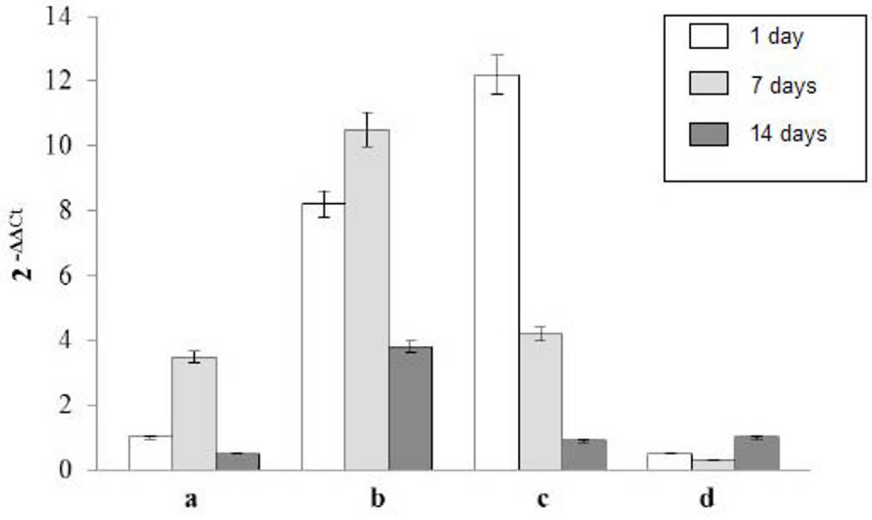

Figure 2. Diagram of relative luxb gene expression level in *P. phosphoreum* colonies during two weeks of growth after different treatments: control (a); UHF EMR at 15 W, 2.45 GHz, 5 min (b); UHF EMR at 15 W, 2.45 GHz, 15 min (c); pre-heating at 42°C (d).

luminous bacteria, their ability to emit light, in particular, and the results obtained in these researches are inconsistent. Induction of light emission by six strains of marine bacteria (belonging to four species: *P. leiognathi*, *P. phosphoreum*, *Vibrio fischeri* and *V. harveyi*) was revealed in response to UV-irradiation and chemical mutagens [25], while a loss in the ability to emit light by luminous bacteria was detected in the presence of toxicants at very low concentrations [26]. The increase of lux gene expression in *P. leiognathi* was found if bacteria grew in the Hg^{2+} environment [27] and the decrease of luminescence with the rising temperature was detected in *Photobacterium* spp. [19]. The effects observed in these studies were species- and strain-specific and depended on the nature of stress factor, its concentration or dose value and duration of treatment.

The influence of two stress factors – UHF EMR and elevated temperature – on *P. phosphoreum* colony morphological and genetic peculiarities was examined in the present research. The results of the performed analyses defined different bacterial stress response on these factors. The elevated temperature caused the decreasing colony morphological parameters and mRNA luxb gene level while irradiation at UHF EMR led to the increase of both analyzed characteristics. But it is worth noticing that we observed enlargement of general colony area in irradiated colonies while area of light zone consisting of metabolically active cells decreased. The heat-shock response is one of the most fundamental responses and found in all living organisms. Heat shock evokes physical and chemical changes in bacterial proteins and membranes. As a response to elevation of temperature, the induction of a large set of proteins (heat-shock proteins, HSPs) occurs [28]. It has been indicated that in bacteria the regulation of heat-shock operons involves a variety of transcriptional activators (the alternative sigma factors) and transcriptional repressors (ROSE and HrcA-CIRCE control elements, for example) [28]. Bioluminescence is a cell density-dependent process of quorum-sensing regulation [29]. Exposure to millimeter waves has been reported to change DNA conformation and thereby gene expression and bioluminescence in high density cultures of quorum-sensing bacteria [30]. Thus, millimeter/submillimeter waves might be involved in a cooperative response within the quorum [31]. Taking into consideration these observations, implementation of electromagnetic fields as quorum- quenching agent has been proposed [32]. But it has been also suggested that expression of genes involved in bacterial luminescence might be controlled by other factors, for example SOS regulon [25]. The difference in stress response revealed in our study and evaluated through the expression of luxb gene may support these assumptions. Besides, effects revealed in our study assumes that UHF EMR effect is caused not only by thermal component but specific factors of non-thermal nature.

Therefore, the results presented in this report indicate that irradiation to UHF EMR affected *P. phosphoreum* growth and stimulated luciferase β-subunit encoding gene expression and its activity depended on exposure duration. It is also likely that control of luciferase gene expression is realized independent of quorum-sensing regulation. Clarification of the mechanism of UHF EMR action on bacterial luminescence is the subject of further studies.

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